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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Organized: New York City, Dec. 10, 1920. Of those at the meeting of organization, the following remain active members: Adolph Berger, Theodor Blum, G. W. Clapp, A. H. Merritt, B. B. Palmer, L. M. Waugh.

Objects (Const., Art. II): The association has been established to promote broadly the advancement of research in all branches of dental science and in the related phases of the sciences that contribute directly to the development of oral health service, and as a whole; and further, to encourage and facilitate cooperative effort and achievement by, and mutual helpfulness among, investigators in all nations in every division of stomatology to the end that dentistry may render cumulatively more perfect service to humanity.

Membership: Total (October, 1958), 938. *Eligibility (Const., Art. III, Sec. 1).*—"Any person who has conducted, and published an account of, a meritorious original investigation in dental science, or in any of the sciences contributory to oral health, shall be eligible to membership in this Association, providing such person conforms to the recognized standards of professional ethics." *Election.*—New members may be elected *only at general meetings of the Association.* Membership in a section is secondary to membership in the Association as a whole. Removal of members from one geographic center to another does not affect their membership in the Association.

Nomination blanks for membership in this Association may be obtained on request from the Secretary-Treasurer.

Endowment fund of the International Association for Dental Research (as of October, 1958): $1,891.01. There have been no expenditures from this fund.

THE JOURNAL OF DENTAL RESEARCH

This JOURNAL is an official publication of the International Association for Dental Research. It is dedicated to the dissemination of knowledge pertaining to the oral cavity, its contents and contiguous parts, and its relation to and effect on the total human organism. The JOURNAL publishes original investigations in these and related fields. Articles are accepted only if they are contributed solely to this JOURNAL.

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All manuscripts and correspondence should be addressed to the Editor: Dr. F. J. Orland; Zoller Dental Clinic, University of Chicago, 950 E. 59th Street, Chicago 37, Illinois.

EDITORIAL VIEWS AND NEWS

The highpoint every year of I.A.D.R. activity is the annual meeting which took place this year in San Francisco, March 19-22. *Highlights of the 1959 Meeting.*—During this 3½ day meeting, there were a total of 279 papers presented verbally. Of this number, 258 were the usual research reports, including 35 from the Dental Materials Group. There were 8 papers in each of two Symposia and 5 presentations under the significant theme of "Impact of Dental Research on the Teaching and Practice of Dentistry." Attendance was good at the entire meeting with a registration of 687.

At the banquet, Hamilton B. G. Robinson, former editor, presented an interesting résumé of the JOURNAL'S history in his Presidential Inaugural Address. This will be published in a future issue. Other points of some significance at this 37th General Meeting were the decisions that there would be no "Read-by-Title" section at the 1960 Chicago meeting; however, a channel for them to appear in print is provided in the section of Research Annotations in the JOURNAL.

Because many members feel it is useful to have abstracts of meeting papers, these are to be made available in mimeographed form before the Chicago meeting. This necessitates an increase in the registration fee and an earlier deadline (Nov. 30, 1959).

The Souder Award.—For outstanding achievement in dental material research, the Wilmer Souder Award was presented to Ralph W. Phillips of Indiana University this year.

The Hatton Award.—It has become traditional to present an award of $100 to the novice researcher who has read the best paper at each annual meeting. Two years ago this event was named the Edward H. Hatton Competition to honor the man whose name it bears for the great part of his 80 years spent in I.A.D.R. activities and in motivating young men to engage in research. At this past meeting, there were 18 such presentations, with the top winners being Richard L. Hoffman (first) and Benjamin F. Hammond (second). Dr. Hoffman, a 1946 graduate of Northwestern, is from the University of Illinois where he is working toward his Ph.D. degree. His presentation was entitled "Formation of Periodontal Tissues Around Subcutaneous Transplanted Hamster Molars." Dr. Hammond, a 1958 graduate of Meharry, is from the University of Pennsylvania where he is also working toward his Ph.D. degree. His presentation was entitled "Submaxillary Saliva as a Culture Medium."

Future Meeting Sites.—Cities for future annual meetings are as listed in the proceedings of the 36th General Meeting (J. D. Res. 37: 956, 1958) with the exception that, for the year 1965, Toronto has definitely been selected. This is an excellent choice since, as an international scientific association, we should meet outside the United States on some occasions.

An Editor's Creed on Conciseness.—One is often impressed (in a negative manner) with overly long research reports whether presented verbally or in manuscript. The following anonymous poetic endeavor, as received from Dr. Dwight J. Ingle, Editor of *Perspectives in Biology and Medicine,* makes a strong, albeit, crude point of such cases.

If you've got a thought that's happy—
Boil it down.
Make it short and crisp, and snappy—
Boil it down.
When your brain its coin has minted,
Down the page your pen has sprinted,
If you want your effort printed,
Boil it down.

Take out every surplus letter—
Boil it down.
Fewer syllables the better—
Boil it down.
Make your meaning plain—express it,
So we'll know—not merely guess it,
Then, my friend, ere you address it,
Boil it down.

Skim it well—then skim the skimmings—
Boil it down.
Trim it, then retrim the trimmings—
Boil it down.
When you're sure 'twould be a sin—to
Cut another sentence in two,
Send it in, and we'll begin to—
Boil it down.

Thus, with these items boiled down, we close.

—FRANK J. ORLAND
EDITOR

THE INFLUENCE OF THE STAGE OF TOOTH DEVELOPMENT ON THE OXYGEN QUOTIENT OF NORMAL BOVINE DENTAL PULP

ALTON K. FISHER, JAMES H. BELDING, JACK S. OPINSKY, AND DANIEL J. SPINELLA

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ACCUMULATING data on the rate of endogenous oxygen consumption in normal bovine dental pulp have revealed less consistent behavior of this tissue than was originally supposed. The mean oxygen quotient first reported for it was 0.56 ± 0.13.[1] A subsequent study in this laboratory produced very similar results (QO_2 0.54 ± 0.15) but indicated that they could be achieved only if relatively high oxygen pressures were maintained.[2] A third study, of preliminary nature, designed to explore the possible effects of various dental drugs and materials on the respiratory behavior of the pulp, revealed what were regarded as rather excessive variations under given experimental circumstances.[3] Continuing work with this tissue showed increasing inconsistency in the mean level of aerobic respiration. A cumulative series of 885 QO_2 determinations produced a mean value of 0.73 ± 0.47. Before further special studies could be undertaken it was necessary that the cause of this great variation be identified.

The possible causes most seriously considered were: limitations of the manometric method, excessive thickness of tissue slices, excessive loading of reaction vessels, bacterial growth in the tissue samples, and degeneration of the tissue during preparation and observation. Detailed analyses of the effects of a series of barometrically determined atmospheric pressures acting simultaneously on all of the flask-manometer systems in the completely assembled and operating Warburg apparatus, but with no tissue in any of the systems, showed that the instrumental error could account for only a small portion of the variation. Calculations based on observed discrepancies between manometers indicated that if the mean QO_2 of a given series of samples of identical activity was only 0.12, the instrumental error could be the basis of a standard deviation of 0.06. Similar calculations based on a hypothetical and identically active series of samples with a QO_2 of 0.69 showed that the manometer error could result in a standard deviation of 0.07, but this degree of variation was very much less than that shown by the tissue actually studied.

This research was supported in part by U. S. Public Health Service Grant No. D-126.
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When the question of suitable thickness for pulp slices was considered, Warburg's formula[4] was used to calculate maximum permissible thicknesses appropriate to the range of QO_2 values previously observed. While comparatively thick slices, 3.66 mm., were allowable for pulp with a QO_2 near 0.50, an attempt had always been made to keep them close to 0.5 mm. in thickness, employing a Stadie-Riggs slicer for this purpose. In order to test the influence of slice thickness and flask overloading on the rate of oxygen consumption, the QO_2 was determined for four series of pulp samples in which these factors were different. The results, presented in Table I, show that slice thickness within calculated permissible dimensions and that flask loads in excess of those ordinarily used in our laboratory exert, at most, an inconsistent effect. There is more reason to believe they were unimportant.

TABLE I
RELATION OF SLICE THICKNESS AND FLASK LOAD TO OBSERVED QO_2

SERIES	NO. OF SAMPLES	APPROX. SLICE THICKNESS (MM.)	MEAN DRY WT. OF TISSUE (MG.)	MEAN QO_2	S. D.	COEFFICIENT OF VARIATION	RANGE
A	28	Less than 0.5	22	0.50	0.18	36	0.16-0.93
B	20	Less than 0.5	75	0.83	0.34	41	0.39-1.54
C	27	1.5-1.0	38	0.79	0.36	45	0.20-1.86
D	40	1.5-1.0	74	0.67	0.24	36	0.30-1.23

The reason for suspecting bacterial growth as a possible factor was the increased oxygen consumption noted in the second and third in vitro hours in some samples previously studied. Because of this, only first hour data have previously been included in our reports. A series of 8 samples of bovine pulp tissue, therefore, were observed for 6 consecutive hours and QO_2 values were calculated for each sample for each hourly period. At the end of the 6 hours, tubes of nutrient broth were inoculated with small quantities of the suspending medium and incubated for 24 hours. A marked turbidity of the broth indicated abundant bacterial growth. The effect of bacterial growth on the QO_2, most noticeable after the fourth in vitro hour, is shown in Table II.

TABLE II
EFFECTS OF BACTERIAL GROWTH ON QO_2 VALUES OF PULP SAMPLES IN KREBS-RINGER-PHOSPHATE SOLUTION

PULP SAMPLES	OXYGEN QUOTIENTS					
	1ST HR.	2ND HR.	3RD HR.	4TH HR.	5TH HR.	6TH HR.
A	1.379	1.401	1.335	1.554	1.620	2.452
B	1.359	1.277	1.277	1.468	1.523	2.175
C	0.747	0.662	0.628	0.900	1.783	3.124
D	0.648	0.278	0.521	0.452	1.495	3.876
E	0.690	1.200	1.540	2.060	4.630	7.370
F	0.470	1.200	0.940	1.200	1.880	7.780
G	1.760	1.360	2.190	2.190	7.030	14.480
H	0.810	0.720	0.810	0.630	2.510	3.320
Mean	0.983	1.012	1.155	1.307	2.818	5.572
S. D.	0.425	0.382	0.514	0.591	1.868	3.916
Coefficient of variation	43	38	44	45	66	70

A similar series of observations was made on pulp samples protected with tetracycline hydrochloride in a 1:100,000 concentration in the Krebs-Ringer-phosphate suspending medium. The nutrient broth, later inoculated with the suspending medium, remained clear after incubation. The QO_2 of the samples varied slightly from hour to hour but the bacterial effect was not observed. The unexpected finding of this experiment was the absence of significant degeneration of respiratory metabolism during the 6-hour period of observation. The results of this series of determinations are presented in Table III. The combined data of Tables II and III are shown graphically in Fig. 1.

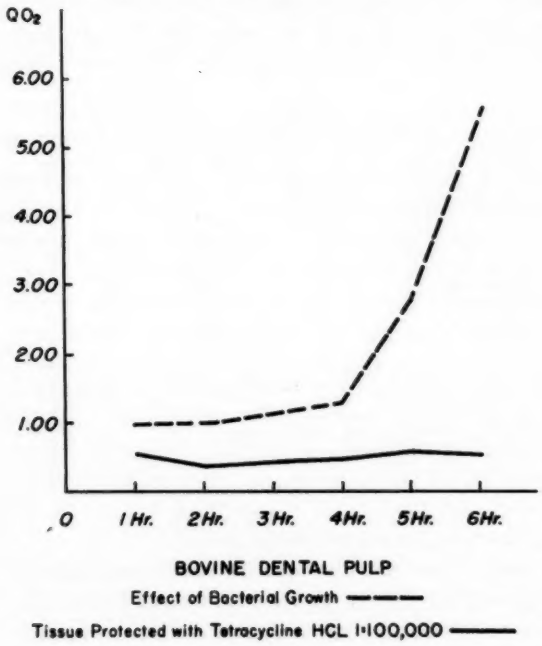

Fig. 1.

In re-examining the first hour data of Tables I, II, and III, it will be noted that QO_2 values for individual samples range from 0.16 to 1.86. Even higher values had been observed on previous occasions but it had been assumed that they were erroneous and were, therefore, excluded. However, since experimentation had shown that suspected technical causes of excessive variation in oxygen consumption were less important than had been supposed, the pulp tissue itself was eventually suspected of greater variability than had previously been recognized. The QO_2 data obtained during the immediately preceding several months by the present workers revealed that some batches of tissue were more active than others. It also became apparent that individual workers had selected certain types of teeth at the abattoir because of the ease of extraction, the slicing characteristics of the tissue, or the volume of pulp required for the day's work. These various features appeared to be related to the developmental status of the tooth.

TABLE III
RESPIRATORY RATE OF BOVINE DENTAL PULP SAMPLES PROTECTED FROM BACTERIAL GROWTH BY TETRACYCLINE HYDROCHLORIDE, 1:100,000

PULP SAMPLES	OXYGEN QUOTIENTS					
	1ST HR.	2ND HR.	3RD HR.	4TH HR.	5TH HR.	6TH HR.
J	0.609	0.581	0.397	0.553	0.737	0.709
K	—	0.379	0.400	0.400	0.505	0.463
L	0.547	0.389	0.447	0.519	0.562	0.562
M	0.728	0.391	0.426	0.444	0.568	0.515
N	0.808	0.602	0.632	0.735	0.799	0.647
O	0.394	0.210	0.303	0.222	0.303	0.303
P	0.499	0.331	0.344	0.317	0.427	0.386
Q	0.356	0.245	0.220	0.233	0.282	0.245
Mean	0.563	0.391	0.396	0.428	0.523	0.479
S. D.	0.153	0.132	0.112	0.128	0.173	0.151
Coefficient of variation	27	34	28	30	33	31

MATERIALS AND METHOD

As in our previously reported studies,[1, 2, 3] molar teeth from freshly killed cattle not more than 2 years old provided the pulp tissue. The extracted teeth were placed immediately in chilled Krebs-Ringer-phosphate solution and taken to the laboratory as quickly as possible. The teeth were divided into three arbitrary classes based on the degree of developmental advancement, designated *Embryonic, Young,* and *Mature,* respectively. Those teeth classed as *Embryonic* included all which showed no root development and hence were actively involved in dentinogenesis. Those classed as *Young* were in some phase of root formation. Teeth classed as *Mature* were those in which root formation had been completed.

The pulps were removed from the teeth and replaced in fresh, chilled Krebs-Ringer-phosphate solution from which they were taken as needed for slicing in a Stadie-Riggs tissue slicer. Slice thickness in this study was approximately 1 mm. Oxygen consumption was measured by the direct method of Warburg as described by Umbreit, Burris, and Stauffer,[5] which was also the case in our previous studies. The suspending medium was Krebs-Ringer-phosphate solution buffered to pH 7.4 and to which tetracycline hydrochloride was added in a 1:100,000 concentration to inhibit bacterial growth. Actual measurement of oxygen consumption was started within approximately 2 hours after the animals had been killed. The water bath was maintained at a temperature of 38° C. The gas phase was oxygen. The gas volume of each flask-manometer system was determined by the method of Lazarow[6] while the sample and suspending fluid were still in the system. After manometric observations were completed, each sliced sample was transferred to aluminum foil weighing baskets, placed in a drying oven at a temperature of 105° C. for 24 hours, and then weighed on a chainomatic balance. The QO_2 was calculated on a dry weight basis only.

RESULTS

A preliminary series of QO_2 determinations were made on a 1-hour basis only on 9 samples each of *Embryonic, Young,* and *Mature* pulp tissue. The

TABLE IV

THE RELATION OF TOOTH DEVELOPMENT TO THE QO_2 OF NORMAL BOVINE DENTAL PULP

	CLASS OF PULP TISSUE											
	EMBRYONIC				YOUNG				MATURE			
HOUR	NO. SAMPLES	MEAN QO_2	S. D.	RANGE	NO. SAMPLES	MEAN QO_2	S. D.	RANGE	NO. SAMPLES	MEAN QO_2	S. D.	RANGE
1	28	2.04	0.39	1.29-2.76	57	0.95	0.24	0.40-1.86	38	0.47	0.15	0.09-0.75
2	28	1.90	0.37	0.86-2.87	34	0.92	0.19	0.38-1.78	26	0.42	0.11	0.21-0.66
3	28	2.25	0.66	1.49-3.56	33	0.97	0.14	0.42-1.70	27	0.46	0.16	0.22-0.88
4	28	2.16	0.57	0.91-3.12	34	1.00	0.32	0.42-1.61	26	0.41	0.14	0.14-0.81
5	25	2.15	0.54	1.39-3.15	32	1.02	0.41	0.38-1.92	25	0.44	0.14	0.24-0.74
6	22	2.12	0.51	1.02-3.12	29	0.99	0.37	0.33-1.88	23	0.39	0.16	0.14-0.71
7	18	2.48	0.51	1.19-3.54	26	1.05	0.35	0.45-1.86	20	0.42	0.14	0.14-0.75
8	18	2.43	0.95	0.98-3.66	24	1.11	0.43	0.41-2.04	20	0.53	0.40	0.24-1.80
9	17	2.53	1.19	1.53-5.88	16	1.11	0.51	0.34-1.98	18	0.42	0.17	0.14-0.74
10	12	2.35	0.77	0.93-4.27	16	1.20	0.53	0.34-2.21	13	0.41	0.17	0.19-0.78
11	12	2.09	0.59	0.72-3.14	16	1.06	0.48	0.33-2.07	13	0.48	0.36	0.50-1.46

consistency of behavior of each class of tissue was greater than anticipated in light of previous experience. Since these results offered the greatest hope of solution of the problem of excessive variations, similar observations were extended to include 28 samples of *Embryonic,* 57 samples of *Young,* and 38 samples of *Mature* pulp tissue. While the QO_2 for all specimens was determined for at least the first hour, the oxygen consumption for one third of the samples was measured at hourly intervals for as long as 11 hours. Hourly determinations were made on 9 samples for 26 hours. Three samples of *Mature* pulp were observed for 28 hours and were still respiring when observations were terminated.

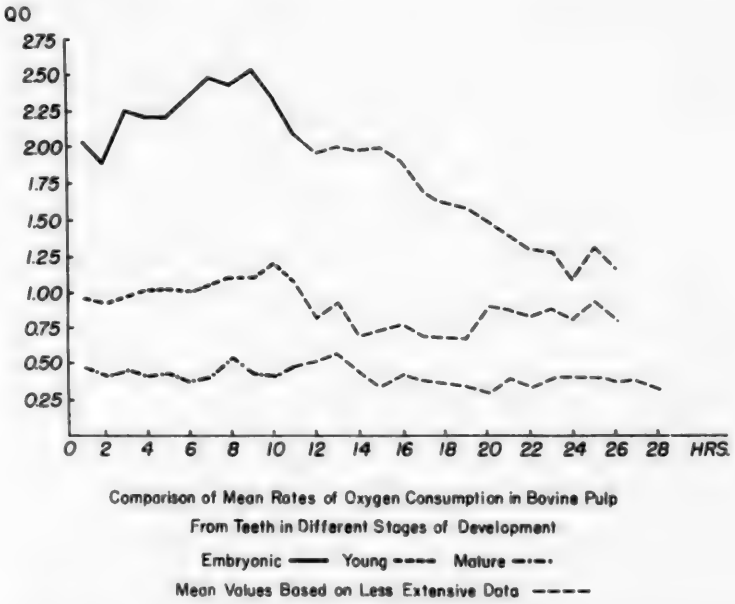

Fig. 2.

The data pertaining to these 123 samples of pulp and representing 802 QO_2 determinations are shown in Table IV. They describe the respiratory behavior of the three classes of tissue through an 11-hour period. This cut-off time was selected for Table IV because it appears to include the period of most normal tissue activity for all three classes. After that time, evidences of metabolic degeneration become apparent although the decline is most noticeable in the more active tissues. Even though the data for observations extended beyond 11 hours are less extensive and the validity of mean QO_2 values, therefore, more subject to question, they are presented graphically in Fig. 2 to illustrate the general comparative respiratory activity of *Embryonic, Young,* and *Mature* bovine dental pulp.

DISCUSSION

The results of this investigation show that the mean rate of oxygen consumption in pulps of bovine molars varies from a relatively high level during

active dentinogenesis to a much lower level after tooth formation has been completed. It is quite likely that similar circumstances prevail in other mammalian teeth except, perhaps, in the incisor pulps of rodents where dentinogenesis is a continuing process. If our data have been interpreted correctly, the QO_2 of rodent incisor pulp should be found to be uniformly high as far as pulp tissue only is considered.

The cause of the declining QO_2 with advancing tooth maturity is not dependent upon decreasing pulp volume since the QO_2 is calculated on a "per milligram of dry weight" basis. It is possible that this change may be due to decreasing cellularity of the pulp since the pulp matrix becomes more fibrous with advancing age. However, we have not yet made comparative cell counts on this tissue and know of no specific information on this point. It is also possible that quantitative changes in the concentration of mitochondria in pulpal cells may occur. The demonstration of mitochondria in active cells with abundant cytoplasm, as in muscle or liver, for example, is relatively simple, but our efforts to show them in pulpal cells have been much less fruitful. These cells, by comparison, have scant cytoplasm with apparently few mitochondria visible by conventional light microscopy. Preliminary examinations by electron microscopy, generously contributed by David B. Scott of the National Institute of Dental Research, have shown that this difficulty is not beyond solution by this method. A third possible cause of decreasing QO_2 may be a shift in metabolic pathways as the tissue completes its most active phase of dentinogenesis. This possibility has not been investigated.

The apparent metabolic durability of pulp tissue in vitro is in seeming contrast with the widely held view that this organ is exceptionally susceptible to the effects of circulatory disturbances. It is emphasized that no nutritive substrate was added to the suspending fluid in any of the work represented by the tabular data of this report. It is well known that tissues such as brain, liver, kidney, and myocardium not only utilize larger quantities of oxygen per unit of time and mass but also demand nutritive support, usually provided as glucose, if the QO_2 is to be maintained on a reasonably constant level. This is not true for dental pulp. It may well be that the matrix of this tisue serves as a nutritional reservoir from which the pulpal cells derive their metabolic needs for many hours after isolation from an active circulation. It is also possible that the respiratory rate is so low that intracellular reserves are sufficient for this period.

Despite the more consistent activity of pulp from different teeth in similar stages of development, considerable variation still exists. This is also observed in other tissues. It undoubtedly reflects variation in physiologic status of the animal from which the samples are obtained as well as variation in local tisue factors. For this reason it may be preferable to employ control tissue from the same animal when testing for the effects of a given experimental influence on pulp, rather than to compare the results with normal mean values obtained from a large series of animals.

SUMMARY

The mean QO_2 of bovine dental pulp was found to be 2.04 ± 0.39, during active dentinogeneses, but was reduced to 0.47 ± 0.15, after dentin formation had been completed. This tissue, generally, has the ability to maintain a reasonably level plane of respiration for as long as 11 hours in vitro without nutritive support. This capacity is greater than that of many of the more active tisues, and it is suggested that the pulpal matrix may function as a nutritional reservoir.

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A DESCRIPTION OF THE MOLAR TEETH AND INVESTING TISSUES OF NORMAL GUINEA PIGS

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THE unique arrangement of the tissues of the molar teeth and supporting tissues of the guinea pig afford investigators an opportunity to study several types of epithelial and connective tissues within a restricted area, under a state of constant synthesis and breakdown. The molar teeth grow continually and are worn off at the occlusal surface at a relatively constant rate. The rate of growth is such that one can view, in a single section, the happenings that have occurred within the tissues during the previous 40 days, and date the happenings accurately. Also, because of the high rate of synthesis and tissue destruction that occurs here, the molar area is a good place to study the unique functions of the periodontal tissues.

Since the results of experimental procedures on tissues cannot be properly evaluated without a clear knowledge of the normal structure and function of the part, and since preliminary observations of guinea pig molars and periodontal tissues indicated differences from the structure as described in earlier reports (Gottlieb and Greiner,[1] Sicher,[2, 3] Fish and Harris,[4] Santone,[5] Wassermann[6]), the present study was undertaken to clarify and supplement the information provided in earlier studies. In addition, since no report was found in the literature which described the increase in size of the molar teeth and the jaws of guinea pigs with age, a map of the normal bone resorption and deposition pattern has been made so that comparisons can be made with the pattern in abnormal, or experimental animals.

MATERIALS AND METHOD

The material for study was taken from 16 guinea pigs, ranging in weight from 190 to 270 grams, which were then maintained in the animal colony on a semi-synthetic diet (Reid and Briggs[7]) for at least 48 days. The animals were decapitated and the heads were fixed in Bouin's solution. The hard tissues were decalcified by the formic acid-sodium citrate method, embedded in paraffin, and sectioned at 5 μ. Sectioning was as follows.

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1. Mesiodistal sections of the left upper and lower jaws and molar teeth were cut approximately parallel to the central long axis of the molars.

2. Cross sections of right upper and lower jaws and teeth were made at three levels: one just above the crest of the interdental bone septum, a second just below the crest of the septum, and a third at the formative ends of the teeth.

3. Buccolingual sections of each upper and lower molar tooth, parallel to the long axis of the tooth, were taken from 3 of the guinea pigs.

Adjacent sections in each plane were stained with the following dyes: hematoxylin and eosin, toluidine blue, van Gieson collagen stain, and Conciere and Friere's silver stain.

In addition, dried skulls of guinea pigs, from 3 days to one year of age, were studied to learn the range in size and curvature of the molars and their occlusal relation.

I. *General Description of Guinea Pig Molar Teeth and Periodontal Tissues.*—

Guinea pigs have continously erupting, laminated molars. Each tooth is comprised of a core of dentin covered by a layer of enamel on all axial surfaces except the lingual of the lowers and the buccal of the uppers (Fig. 2). Viewed from the occlusal aspect, two deep folds penetrate the substance of each tooth, one entering from the buccal side and the other from the lingual. These folds have a thin enamel lining and contain cartilage which is cemented to the enamel surface. The apical two thirds of the molar is embedded in a bony alveolus, the walls of which follow the irregular contours of the tooth.

One end of the principal fibers of the periodontal membrane is embedded in the alveolar bone and the other, because of the varied composition of the tooth surfaces facing the membrane, is attached to either enamel, dentin, or cartilage (Fig. 2). In addition, transeptal fibers extend from tooth to adjacent tooth in the arch, occlusal to the interdental bone crest (Fig. 1).

Fish and Harris[4] have estimated that guinea pig molars, under conditions of normal attrition, are completely replaced by new tooth structure every 40 days. Continuous replacement of tooth and periodontal tissues occurs by means of a persistent enamel organ, surrounded by a primitive mesenchymal type of tissue at the apex of the tooth.

II. *The Development of Dental and Supporting Tissues.*—

Because the structure of the supporting tissues of the guinea pig molar varies, depending on the area of the tooth to which it is attached, it is simpler to describe the histogenesis and microscopic structure of the tooth before discussing the formation and structure of the supporting tissues.

Enamel: The enamel organ is best described as it appears at the mesial or distal side of the tooth in a longitudinal section rather than in the central area where the cartilage makes the histologic picture more complicated (Figs. 1 and 3). At the apex of the molar, the enamel organ consists of four histologically distinct layers of epithelial cells, i.e., and ill-defined outer enamel

epithelium, a stellate reticulum, a stratum intermedium, and an inner enamel epithelium. At the most apical point of the organ, the outer and inner layers are continuous, forming the cervical loop (Figs. 1 and 3).

Enamel begins to form a short distance occlusal to the cervical loop and, at a point about one third of the distance from the loop to the crest of the

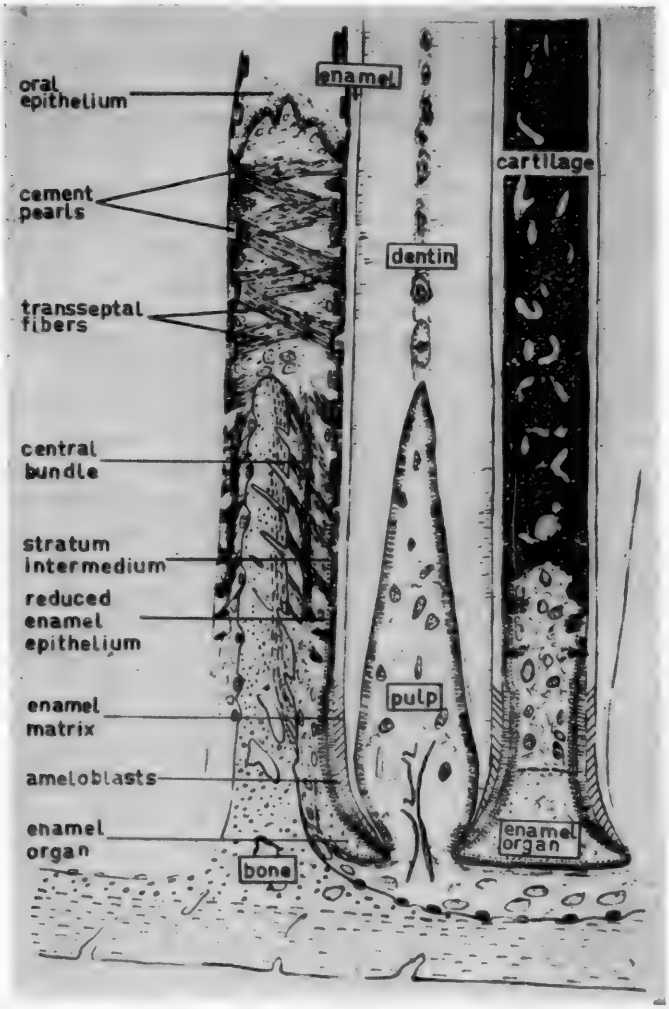

Fig. 1.—Diagrammatic drawing of a longitudinal, mesiodistal section to show half of a lower guinea pig molar and the interdental periodontal tissue between it and the adjacent tooth.

interdental bone, maturation becomes completed. From this point occlusally no enamel is visible in demineralized sections. The enamel organ, bounded on its outer aspect by fine, reticular fibers, becomes reduced in thickness approximately halfway along the immature enamel. The outer enamel epithelium

LINGUAL

MESIAL

Fig. 2.—Low-power photomicrograph of a cross section of the lower left, first, and second molars of a normal guinea pig, at a level just above the crest of the interdental bone septum. Note the bone deposition on the lingual surface of the alveoli. (Toluidine blue stain.)

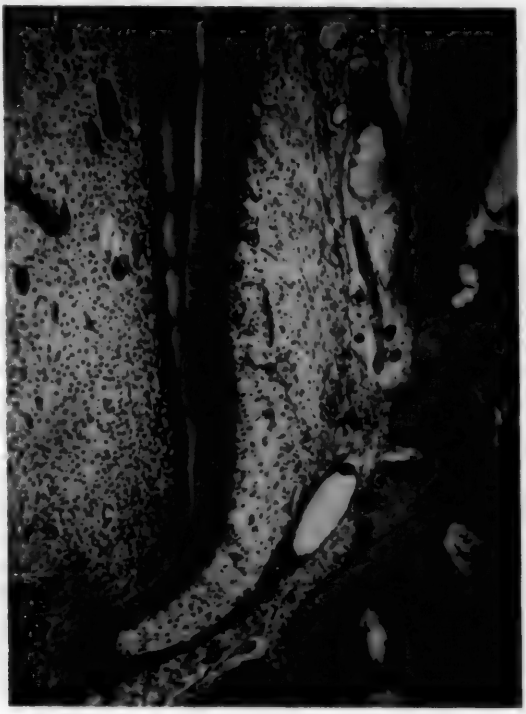

Fig. 3.—Photomicrograph of a longitudinal section through the enamel organ at the mesial edge of a lower right guinea pig molar. (Hematoxylin and eosin stain.)

and stellate reticulum disappear, and loose areolar connective tissue occupies the area between bone and the enamel matrix (Figs. 1 and 3).

In the region of the cervical loop, cells of the inner enamel epithelium differentiate into the tall columnar ameloblasts which produce enamel matrix. These cells may be identified all along the side of the tooth up to the oral mucosa but their appearance varies depending on their location. When first differentiated, they appear as a row of tall cells with the nucleus at various levels in the cell (Figs. 1 and 3). As enamel matrix production begins, the nuclei migrate toward the cell border away from the secreting ends. When

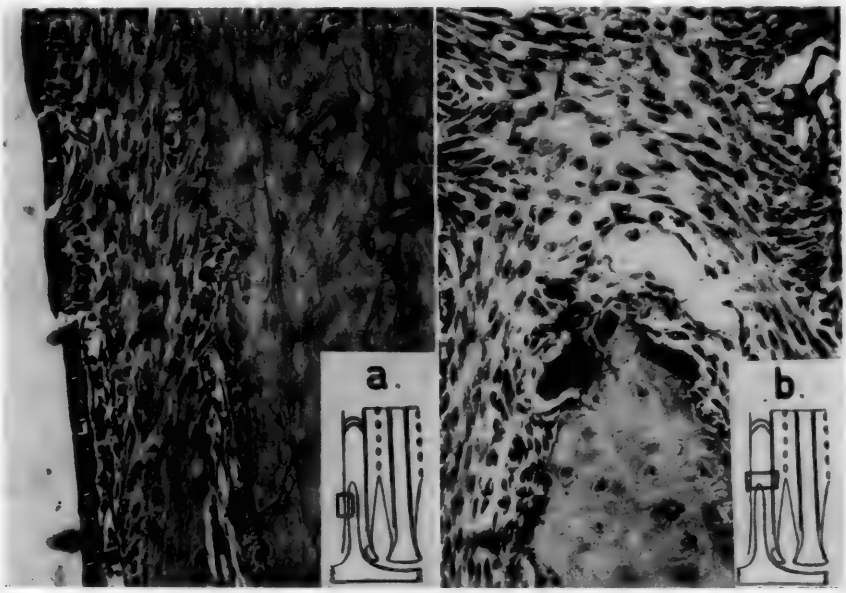

Fig. 4.—*a*, Longitudinal section of the periodontal tissue between the enamel surface and the interdental bone. One layer of reduced ameloblasts, one layer of stratum intermedium cells, capillaries, and loose connective tissue occupy the area between cement pearls. Note the arrangement of the principal fiber bundles. (Hematoxylin and eosin stain.) (Refer to small diagrams for orientation.)

b, Longitudinal section of the interdental periodontal tissue just above the bone crest. Note the pale staining intercellular substance and the abundant cytoplasm of the young fibroblasts. (Hematoxylin and eosin stain.)

enamel maturation is completed, the ameloblasts become reduced in length to low columnar cells. The reduced ameloblasts remain attached to the enamel by the primary enamel cuticle except at the sites of attachment of the periodontal fibers to enamel (Fig. 4, *a*). This arrangement is maintained until just below the oral epithelial attachment where the cells become further reduced in length (Fig. 4, *d*), and then pass through the epithelial attachment, still fixed to the enamel surface. In the oral cavity, they are lost and only the thin cuticle remains.

Stratum intermedium cells also migrate occlusally in conjunction with the ameloblasts and the erupting tooth. They first appear as a single, flattened layer of cells, between the outer enamel epithelium and the stellate reticulum

(Figs. 1 and 3). At the cervical loop, they divide and form several layers of cuboidal or triangular cells with very little cytoplasm. As enamel matrix production begins, the cells exhibit an increase in cytoplasm and a condensation of nuclear chromatin granules. At this site, the cell borders of the ameloblasts adjacent to the stratum intermedium are distinct, but they are not as easily seen at the level where enamel maturation begins. When enamel maturation is completed, the stratum intermedium becomes reduced to a single layer of cells (Fig. 4, *a*) which is carried occlusally, accompanied on one side by reduced ameloblasts, and on the other side by a highly vascular, loosely arranged, connective tissue (Figs. 4, *a* and 4, *b*). The dense fiber bundles of the periodontal membrane interrupt these cell layers to attach to the teeth via the cement pearls. This appearance is maintained to the level on the tooth

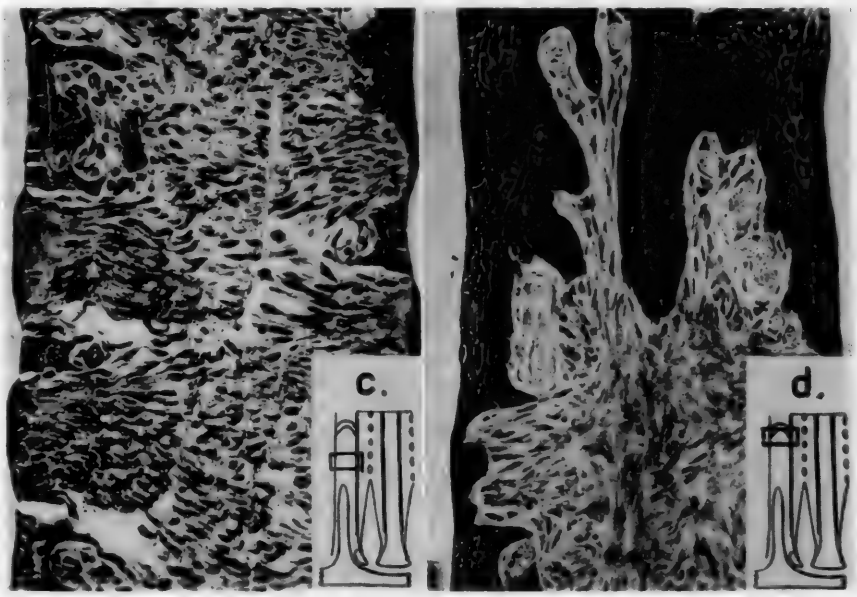

Fig. 4.—Cont'd. *c*, Longitudinal section of the periodontal membrane midway between the interdental bone crest and the oral epithelium. Note that the stratum intermedium cells on the left side have just begun to proliferate while those on the right side have divided to form several layers of cells. (Hematoxylin and eosin stain.)

d, Longitudinal section of the interdental periodontal membrane just below the oral epithelial attachment. The stratum intermedium cells are beginning to cover the cement pearl at the lower left. (Hematoxylin and eosin stain.)

at, or just beyond, the mid-point between the interdental bone crest and the oral epithelium. Here, the cells of the stratum intermedium begin to proliferate and invade the adjacent connective tissue (Fig. 4, *c*). The cells between the basal mitotic layer and the reduced ameloblasts become similar in histologic appearance to the stratum spinosum or prickle cell layer of the oral epithelium with which they eventually merge (Fig. 4, *d*).

Dentin: The process of dentin formation in the guinea pig molar is similar to that in other mammals and needs only a brief description. At the

permanently open apex of the molar, odontoblasts differentiate from the mesenchymal cells of the pulp (Figs. 1 and 3). As the tooth erupts, the usual sequence of dentin formation takes place: formation of predentin with its matrix of Korff's fibers, calcification within the matrix, and migration of odontoblasts in a pulpal direction. Continued deposition of dentin leads to complete closure of the pulp approximately halfway from the apex to the occlusal surface of the tooth. From this point to the occlusal surface, some degenerated pulpal cells remain embedded in the irregular dentin in the central core of the tooth (Fig. 1).

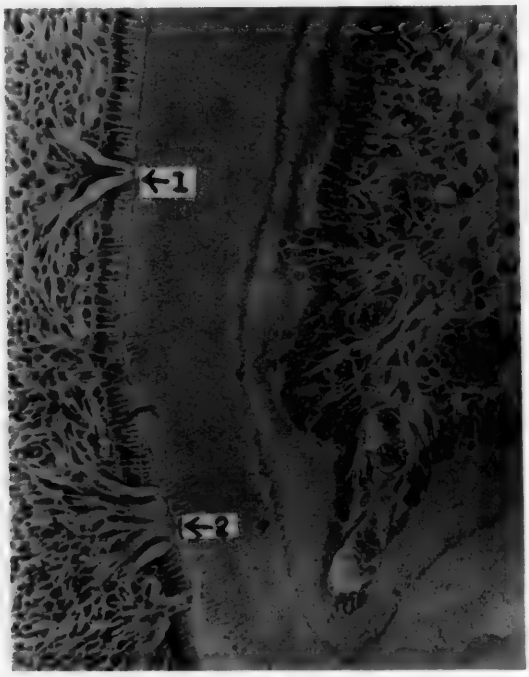

Fig. 5.—Photomicrograph of a cross section of a guinea pig molar at a level just below the region where enamel maturates, to show the beginning of attachment of cement pearls to enamel. The fibroblasts have just penetrated the reduced ameloblasts at *1*. Some enlargement of the attachment has ocurred at *2*. Enamel matrix is still present. (Hematoxylin and eosin stain.)

Cementum: As has been mentioned above, the periodontal fibers of the guinea pig molar are attached to three types of tooth substance: enamel, dentin, or cartilage, and the type of attachment (e.g., cementum) varies in each case.

Attachment to enamel occurs as follows: approximately at the level on the tooth where enamel maturation occurs, a spear-shaped aggregation of fibroblasts appears in the relatively undifferentiated connective tissue of the dental sac between bone and tooth. These cells wedge their way between the stratum intermedium cells and the reduced ameloblasts until they reach and become fastened to the enamel surface (Fig. 5). Almost immediately, the aggregation of cells enlarges, collagen fibers form, and the ground substance between

the newly formed fibers becomes calcified forming the cement pearl (Figs. 1 and 4, *a*). Later, in the region just below the alveolar bone crest, an additional layer of noncellular cementum covers the cement pearl and spreads out on either side of it (Figs. 1 and 8). This secondary layer is related to the attachment of additional collagen fibers to the tooth surface. Just below the oral epithelium where the periodontal fibers disappear, the cement pearls, still attached to enamel, pass through the epithelium (Fig. 4, *d*). In the oral cavity, the cement pearls appear, in demineralized sections, to be linked together by the enamel cuticle (Fig. 1).

Periodontal fibers become attached to dentin surfaces (on the lingual surface of the lower molars and the buccal of the upper molars) at a level closer to the tooth apex than they do on enamel surfaces since no interfering

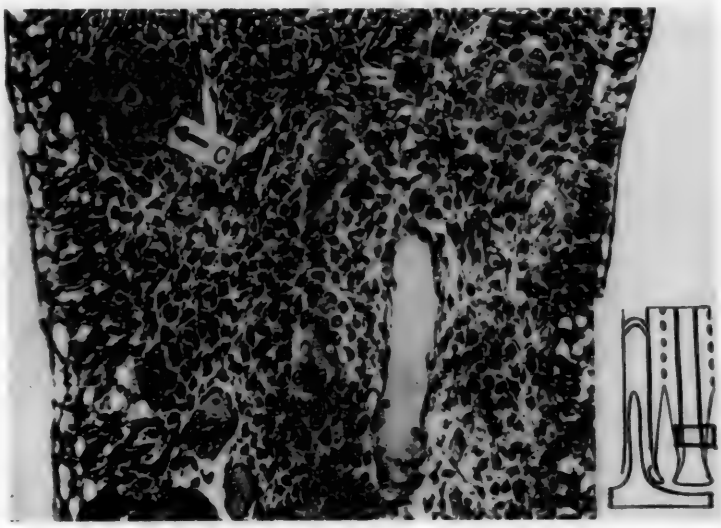

Fig. 6.—Photomicrograph of a longitudinal section of a guinea pig molar occlusal to the enamel organ in the folds of the teeth to show the development of cartilage. Note the cartilage forming in growth centers (*C*) in the vascularized connective tissue. (Hematoxylin and eosin stain.) (Refer to small diagram for orientation.)

enamel organ is present. Attachment takes place through the medium of noncellular cementum. Irregular, secondary, noncellular cementum is deposited as the tooth erupts, and its deposition is apparently related to the presence of a layer of cells with round, open-faced nuclei, which are located between fibers and adjacent to the cementum surface. The periodontal fibers become detached from cementum as they reach the oral epithelium.

The attachment of periodontal fibers to cartilage is intimately related to the formation of cartilage itself. The cartilage within the folds of the tooth originates from the connective tissue occlusal to that segment of the enamel organ found under each fold of the tooth (Fig. 1). Immediately occlusal to the site within the folds at which enamel maturation occurs, cells from the mesenchyme penetrate the reduced ameloblast layer and cartilage matrix forms,

but unlike the cement pearls, chondrocytes become embedded in lacunae within the matrix. Isolated nodules of cartilage also form in the mesenchyme between adjacent enamel surfaces (Fig. 6), and it is at this stage that the periodontal fibers at the open ends of the folds, on the buccal and lingual sides of the tooth, become attached to the cartilage matrix (Fig. 7). Just occlusal to this area, the space between enamel surfaces is bridged with cartilage containing disintegrated vascular inclusions (Fig. 1). Reduced ameloblasts disappear completely. As cartilage nears the occlusal surface during tooth eruption, the chondrocytes die, and the cartilage wears off at the occlusal surface along with the other dental structures.

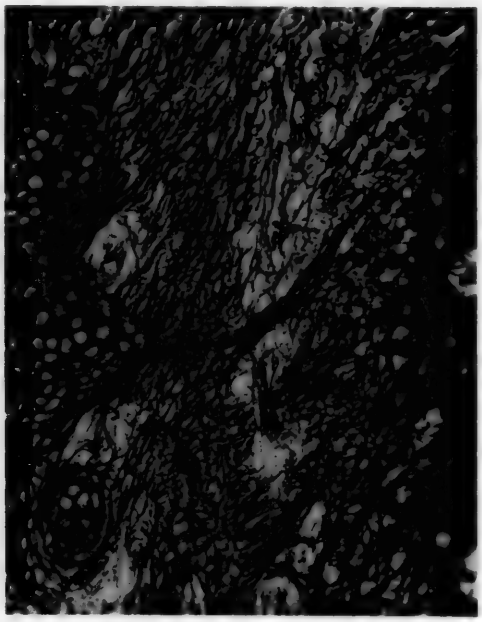

Fig. 7.—Photomicrograph of a buccolingual section of a guinea pig molar showing the attachment of periodontal fibers to cartilage at the entrance to folds. (Silver stain.)

Principal fibers of the periodontal membrane: Just as a mechanism for continuous production of the hard dental tissues must be provided in the guinea pig molar, there must also be a means whereby periodontal fibers are continually replaced as they are lost because of tooth eruption.

There are three main sites of active collagen fiber formation within the periodontal membrane—one at the site of recently matured enamel, a second in the perpendicular bundle of fibers in the middle of the membrane between the tooth and the alveolar wall, and a third just above the crest of the interalveolar bone septum. A fourth, less active site lies along the axial surfaces of the alveolus where bone is being remodelled.

The enamel organ is limited to an area extending about halfway up the organic enamel matrix (Figs. 1 and 3). The dental sac, occlusal to the enamel

organ, is a highly vascular, loosely woven, connective tissue composed largely of reticular fibers and connective tissue cells (Fig. 1). At the point where cement pearls first form, collagen fibers begin to bridge the gap between enamel and alveolar bone (Figs. 1 and 5). This is the first site of significant fiber formation.

Just above this area, the collagen fiber bundles slant upward from cement pearls and downward from the wall of the alveolus to intermingle with a central, perpendicular bundle of fibers (Figs. 1 and 4, *a*). There is general agreement that the interchange of fibers, which is necessary to support the continuously erupting tooth, takes place in, or at the surface of, this central bundle (Sicher,[2] and Santone[5]). Observations made in this study corroborate this.

Fig. 8.—Longitudinal section of the periodontal membrane midway between the oral epithelium and the interdental bone crest. Dense collagen bundles pass directly from a cement pearl on one tooth to two pearls on the adjacent tooth. Secondary cementum is deposited on the surface and at each side of the pearls. (Silver stain.)

The third region of active collagen formation lies immediately above the crest of the interdental bone septum (Figs. 1 and 4, *b*). The fibers are loosely woven, and stain lightly with silver and collagen stains at this site. Most of the intercellular substance is of the amorphous variety. The fibroblast nuclei are plump and ovoid, their cytoplasm is abundant, and numerous blood vessels are present. This sudden transition from intensely stained, dense collagen fibers between bone and tooth, to pale staining ground substance just above the bone crest, suggests that new fibers form in this area. Slightly

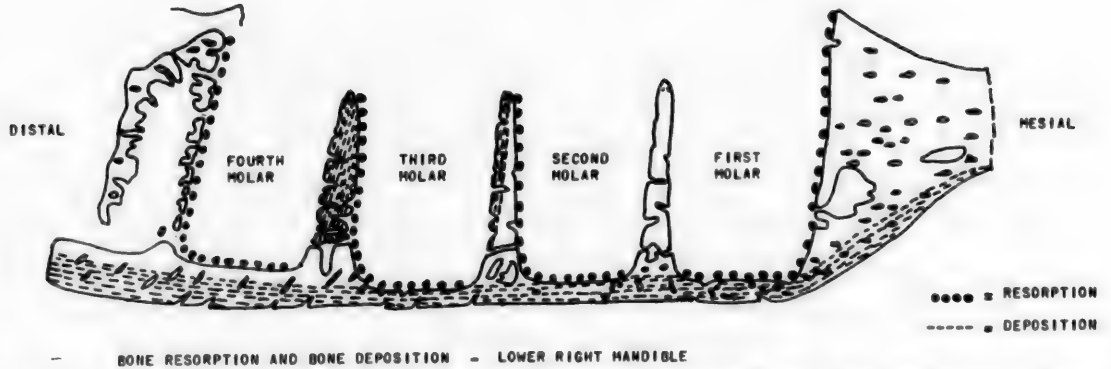

Fig. 9.—Diagrammatic drawing of a longitudinal mesiodistal section of the lower right mandibular bone of a guinea pig. Note the bone resorption and deposition patterns around each tooth socket and along the lower border of the mandible.

occlusal to this region, and continuing to the oral epithelium, intensely stained, dense collagen fiber bundles pass directly from a cement pearl on one tooth to two cement pearls on the adjacent tooth (Figs. 1 and 8). Elongated fibroblast nuclei may be seen between the fibers. Immediately below the oral epithelium the fibers become detached from cement pearls and disappear (Figs. 1 and 4, *d*). The fourth and less active site of fiber formation occurs along the axial surface of the alveolar bone (Fig. 4, *a*). Slow remodelling of the bone surface opens up tunnels into the underlying bone. Collagen fibers grow into the spaces and appositional growth on the side walls of the tunnels encompass the fibers.

The change over of periodontal fibers attached to dentin and cartilage is more difficult to trace since the fiber bundles are less discrete. However, the attachment to the alveolar bone also consists of pendant bundles of collagen fibers embedded in the bone. These in turn are continuous with a perpendicular bundle of fibers midway between the bone and cartilage or dentin. From the perpendicular bundle to dentin, isolated fibers extend almost at right angles to the tooth surface and in their course they are diverted around the cementoblasts. The fibers then branch out as they enter the cementum. The fibers which extend to the cartilage from the central bundle are themselves arranged in bundles. It is likely that they penetrate the cartilage matrix (Fig. 7) but, in some silver-stained sections, they appear to be masked by a cementing substance on the cartilage surface. Numerous capillaries are found adjacent to the cartilage and between fiber bundles.

The fibers underlying the apices of the molars are reticular in structure and their function is the support of the large, thin-walled blood vessels and nerves in that region. They are more densely packed at the foramen and under the outer enamel epithelium. No collagen fibers are present.

III. *The Pattern of Bone Resorption and Deposition in Guinea Pig Jaws.*—

The bone resorption pattern was charted by recording the distribution, size, and number of the osteoclasts in sections from comparable areas in the jaws of each animal. Bone apposition was also recorded in the same charts. New bone was clearly recognizable in sections stained with toluidine blue because of its pale staining (Fig. 2).

Two main factors influence the bone deposition-resorption pattern by affecting the direction and amount of the movement of guinea pig molars through alveolar bone. First, the teeth increase both in length and diameter, as the animal ages. Second, all of the molars curve buccolingually. The upper molars are concave toward the buccal surface and the lower molars are concave toward the lingual surface (Fig. 11), and this curvature decreases progressively from the first to the fourth molar in both jaws. Occlusal forces cause a tipping of the teeth in their sockets.

Resorption occurs along the mesial surface of the alveolus of the first molar, and on the distal surface of that of the second, third, and fourth molars, but none is evident on either side of the septum between the first and second molars (Figs. 9 and 10). Osteoclasts are always present along the bone surface underlying each tooth apex (Fig. 9).

Deposition is evident along the lower border of the mandible and at the crest of each interdental septum, indicating an increase in socket depth as the teeth continued to grow in size. Some bone deposition is visible on both sides of the septum between the first and second molars. More is evident on the distal side of the septum between the second and third molars, and still more

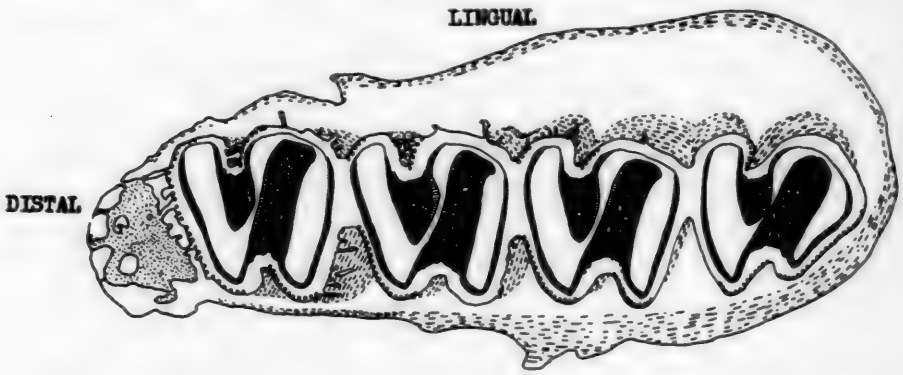

Fig. 10.—Diagrammatic drawing of a cross section of the lower right molar teeth and jaw at a level just above the interdental bone crest. Note the pattern of bone resorption (.....) and bone deposition (_____) around each tooth.

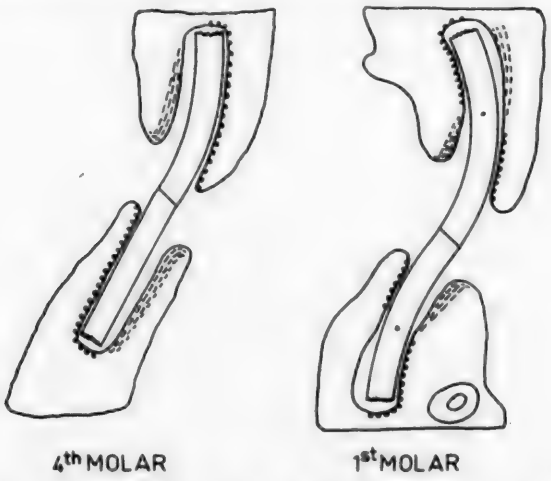

Fig. 11.—Diagrammatic representation of a buccolingual section through the opposed first molars (*right*) and fourth molars (*left*). The resorption (.....) and deposition (_____) of bone is directly related to the curvature of the teeth.

on the distal surface of that between the third and fourth molars (Figs. 9 and 10). On the surfaces of the septa, the incremental layers are deposited along the whole length of the socket parallel to its surface, indicating a bodily movement of the teeth through the bone, rather than a tipping motion (Fig. 9). As the bone pattern suggests, the second molar increases in diameter and

pushes the third molar in a distal direction. The fourth molar must move a greater distance to the distal to accommodate the expanding second and third molars.

At the buccal and lingual margins of the teeth, the pattern of bone deposition and resorption is related primarily to tooth tipping caused by occlusal forces acting on the curved teeth (Figs. 10 and 11) and, to a lesser extent, to the increase in tooth diameter with age. The pattern was traced from cross sections of the teeth and jaws. (To avoid confusion, it should be noted that the interdental bone crests of guinea pigs are saucer-shaped rather than bluntly pointed as in man.) In the mandible, resorption occurs along the buccal alveolar margins. The amount of bone deposited decreases from first to third molars. In the maxilla, the pattern is reversed, i.e., resorption occurs on the lingual and deposition occures on the buccal surfaces.

The influence of the occlusal forces on the resorption-deposition pattern is illustrated diagrammatically in Fig. 10. In the maxillary first molar, the rotation fulcrum is at a point approximately halfway from the alveolar bone crest to the apex of the tooth. Resorption thus occurs occlusal to the fulcrum on the lingual side, and apical to the fulcrum on the buccal surface. The deposition pattern is just the reverse, i.e., it takes place on the buccal surface occlusal to the fulcrum and on the lingual surface apical to the fulcrum. The pattern in the mandibular bone is the reverse to that in the maxillary bone, because the upper and lower molars are curved in a reverse direction to one another.

Because the fourth molars are less curved than the first molars, and thus their fulcrum is below the apex, resorption occurs along the entire lingual margin of the maxillary alveolus, and to the buccal margin of the mandibular alveolus (Fig. 11).

DISCUSSION

Little has been written in the past about the behavior of the residual cells of the guinea pig enamel organ (reduced ameloblasts and stratum intermedium) as they are carried occlusally with the erupting molars. Johnson and Bevelander[8] studied the erupting incisors in successive pig embryos and observed that the stratum intermedium begins to proliferate just prior to fusion of the developing tooth with the oral mucosa. Santone[5] noted the proliferating islets of cells but he failed to recognize that they had their origin in the stratum intermedium. In addition, his suggestion, that the epithelial cells play some part in the dissolution of periodontal fibers when they reach the oral epithelium, is open to question. It seems more probable that the fibers disappear first and the stratum intermedium cells proliferate into the space created. It is interesting to note that stratum intermedium cells from the enamel organ retain the capacity to divide at this high level on the tooth.

Santone[5] and Sicher[2] described fiber formation in the periodontal membrane at the level at which enamel maturation occurs, and above this in the area between the interdental septum and the enamel surface of the molars. The slow remodelling of the surface of the alveolus in the latter area occurs partly

to permit the attachment of new periodontal fibers. However, it should be noted that the interdental septa, between the second and third and the third and fourth molars, move distally with growth, and much of the alveolar remodelling is because of this.

Fibers have also been found to form across the crest of the interdental septum, and they must be carried occlusally as the molars erupt because there is no break in the continuity of the interdental bundles.

Although Santone[5] believed that cartilage plays no part in suspending the molar in its alveolus, it is clear that fibers do extend between, and are embedded in the cartilage and the alveolar wall.

Sicher[3] described a hammock ligament of collagen fibers which was suspended by the side walls of the alveolus and crossed beneath the apices of all continuously erupting rat incisors and guinea pig incisors and molars. From this, it was postulated that the expanding cells of the pulp exerted pressure on the hammock ligament and an eruptive impetus was transmitted to the tooth by means of the collagen fibers suspended in the side walls of the alveolus. In addition, the hammock ligament prevented the resorption of bone beneath the tooth apex.

In this study, no collagen fiber bundles were present beneath the apices of the molars, and there was no substantial attachment of fibers to the walls of the alveoli. Bone resorption occurred under each molar and bone was added to the inferior border of the mandible. If the pulpal cells do impart any eruptive force to the molars, this force is partly resolved in resorbing the underlying bone.

The interpretation of the normal bone resorption and deposition patterns around the socket walls, and beneath the apices of the molars, may be of some value to those who wish to use the guinea pig to assess bone changes due to nutritional deficiencies, hormonal disturbances, and various forms of occlusal stress.

SUMMARY

A description of the gross and microscopic structure of guinea pig molars and their supporting tissues has been presented, together with an account of the normal physiologic changes which occur with age and function. The report is based on a study of sections of the teeth and jaws of 16 normal guinea pigs, and of dried skulls of animals from 3 days to one year old. The points of divergence between this and previous descriptions, and of information in addition to that previously reported, are listed below.

As the molar teeth erupt, carrying with them reduced ameloblasts and stratum intermedium cells, the latter proliferate just before they reach the basal cells of the oral epithelium and assume the microscopic appearance of cells of the intermediate and basal layers of a stratified squamous epithelium. As eruption proceeds, these cells blend with the deeper layers of the oral epithelium.

The nature of the attachment of fibers of the periodontal membrane to the cartilage portion of the tooth has been described.

Secondary cementum forms over, and at each side of the cement pearls, in order to attach additional fibers.

The heavy collagen bundles, which form the interdental fibers of the periodontal membrane, are produced in the area immediately occlusal to the crest of the interdental septum, are carried occlusally with the teeth to which they are attached, and disappear just below the oral epithelium.

The pattern of deposition and resorption of the bony walls of the alveoli has been described. This pattern is the result of two main factors which dictate the direction and amount of movement of the molar teeth through the jaws: first, all of the molars increase both in length and diameter as the animal ages; second, the teeth are curved along their long axes, and the curvature decreases from the first to the fourth molar. The direction of the occlusal force on the teeth causes them to tip in their sockets, and the amount and rotation are related to the tooth curvature.

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THE EFFECTS OF ASCORBIC ACID DEFICIENCY ON THE TEETH AND PERIODONTAL TISSUES OF GUINEA PIGS

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INTRODUCTION

MICROSCOPIC alterations of teeth and jaws resulting from an ascorbic acid deficiency in guinea pigs have been reported in the literature, although the reports are not in complete agreement as to the nature of the alterations. For example, there has not been complete agreement about the effect of severe scurvy on collagen fibers. McLean, Sheppard, and McHenry[1] reported no observed imperfections in collagen fibers under such circumstances, although Glickman[2] stated that collagen fibers broke down. The reports of Wolbach and Howe[3] and Ham and Elliott[4] agreed that collagen fibers failed to form, but did not agree as to whether this was due to disturbances in the fibroblast cells or in the ground substance. Bourne,[5, 6] in his reviews of the relationship of ascorbic acid to wound healing, concluded that ascorbic acid was necessary for collagen fiber formation, but that its role in fiber maintenance was uncertain.

In each of these earlier investigations, basal diets were used in which the exact amount of the various food elements could not be controlled. Diets were composed basically of cereal grains, protein, and a salt mixture and, in most instances, some of the ingredients were heated to destroy retained ascorbic acid, a procedure which may have destroyed other heat-labile, but essential, food factors. Therefore, alterations found in the tissues following the feeding of such a diet may not have been due to an ascorbic acid deficiency alone. In addition, in several of the studies, orange juice or cabbage was used as a source of ascorbic acid in the control diet. This usage could have led to an additional source of error, since it is now known that the amount of the vitamin in these tissues varies in amount (see Fish and Harris,[7] Ham and Elliott,[4] and Wassermann[8]).

Thus, the lack of agreement in some of the earlier reports may have resulted from unavoidable lack of control in the experiments. If this were the situation, then it was felt that some resolution of these apparent controversies should result if a basal semisynthetic diet of known composition, pure L-ascorbic acid supplements, and pair-feeding methods were used, which factors

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would rule out the possibility of pathologic alterations resulting from either other dietary deficiencies, or from variations in the amount of ascorbic acid administered to the animals. In addition, it was felt that further study of the periodontal tissues under conditions of ascorbic acid deficiency could lead to greater understanding of the nature and function of these tissues.

MATERIALS AND METHODS

Guinea pigs, approximately 5 weeks old and ranging in weight from 190 to 270 grams, were maintained on the semisynthetic basal diet of Reid and Briggs.[9] They were assigned at random to one of four groups, depending on the daily ration of ascorbic acid fed to the animals, and the length of time of the experimental feeding period (Table I).

TABLE I
DISTRIBUTION OF GUINEA PIGS ACCORDING TO THE AMOUNT OF ASCORBIC ACID FED PER DAY, THE LENGTH OF THE EXPERIMENTAL FEEDING PERIOD, AND WHETHER PAIR-FED OR FED AD LIBITUM

GROUP NUMBER	NO. ANIMALS	ASCORBIC ACID MG./DAY/ANIMAL	EXPERIMENTAL PERIOD (DAYS)	TYPE OF FEEDING
I	5	0.0	24	Ad libitum
IIa	6	0.4	48	Ad libitum
IIb	6	5.0	48	Pair-fed to IIa
IIc	6	5.0	48	Ad libitum
IIIa	7	0.4	75	Ad libitum
IIIb	7	5.0	75	Pair-fed to IIIa
IIIc	7	5.0	75	Ad libitum
IV	5	0.4	48	Ad libitum
		then 5.0	27	

Group I was completely deprived of ascorbic acid for 24 days in order to study the effects of severe scurvy on the tissues. Group II was divided into three subgroups, IIa, IIb, and IIc. Group IIa animals received 0.4 mg. of ascorbic acid daily for 48 days, so that the effects of moderately severe scurvy could be observed. As controls, Group IIb animals were pair-fed to Group IIa, but received 5.0 mg. ascorbic acid per day, and Group IIc received 5.0 mg. ascorbic acid per day and was fed ad libitum for the experimental period. Group III was arranged similarly to Group II, except that the animals were kept on their various feeding arrangements for 75 days rather than 48 days. The pigs in Group IV were maintained for a period of 48 days on a daily ration of 0.4 mg. of ascorbic acid, and then fed 5.0 mg. per day for the next 27 days to see if any pathologic alterations caused by the reduced ascorbic acid feeding could be reversed when an optimal amount was fed. Animals in either of Group IIc or IIIc were considered as controls for those in Group I.

All the constituents of the basal diet were freshly mixed in a food blender every 3 days and refrigerated between feedings. The diet was dispensed to the animals in half-pint penny-lever tins with an opening of 4.5 cm. at the top. Depressed rims on the tins reduced spillage to a minimum. Distilled

water was offered the animals in unrestricted quantities. The daily ration of ascorbic acid for each group of animals was administered orally in 0.5 c.c. of distilled water, using accurately equilibrated syringes.

In those instances where pair-feeding was carried out (Groups IIa and IIb and IIIa and IIIb), each animal's daily ration was weighed, the unconsumed food weighed the following day, and the pair-fed animals were given the same amount of food that their mates had received the previous day.

The animals were weighed every second day and the weights recorded. At the termination of each experimental period, the guinea pigs were decapitated and the heads fixed in Bouin's fixative. Tissues were demineralized using the formic acid—sodium citrate method, embedded in paraffin, and sectioned at 5 μ. Jaws and teeth were cut in three planes of section, both mesiodistal and buccolingual, parallel to the long axis of the teeth, and cross sections were made at a right angle to the long axis of the teeth at a level just occlusal to the interdental bone septum. Adjacent sections were stained with the following: hematoxylin and eosin, toluidine blue, van Gieson collagen stain, and Conciere and Frere's method of silver staining.

RESULTS

Gross Observations.—

All guinea pigs which were fed diets containing less than 5.0 mg. of ascorbic acid per day demonstrated a hind limb paralysis, and the severity of the paralysis related directly to the reduction of dietary ascorbic acid. Group I animals, whose diet was completely deficient in the food element, developed the most severe paralysis after about 14 days on the diet. All animals receiving 0.4 mg. ascorbic acid per day (Groups IIa, IIIa, IV) developed a similar but less severe paralysis which was not apparent until they had been on the diet about 20 days. Group IV animals completely recovered from their paralysis a day or two after being placed on the diet containing 5.0 mg. ascorbic acid per day. All of the control animals, including those pair-fed, remained alert and active throughout the period of the experiment.

A graph illustrating the weight record of the animals is shown in Fig. 1. The graph includes the record of the guinea pigs from Group I, the three subdivisions of Group III, and of Group IV. The weights of animals from Group II closely paralleled those of Group III and have not been included. It can be seen that the pigs on the diet completely deficient in ascorbic acid (Group I) gained weight normally for a period of about 8 days after being placed on the diet. From the fourteenth day onward, they lost weight rapidly and steadily until at sacrifice on the twenty-fourth day of feeding, they weighed considerably less than at the beginning of the experiment.

Group III illustrates the alterations in weight gain brought about by feeding a less-than-optimal amount of ascorbic acid in the diet, and by pair-feeding with such a deficient group. Group IIIc animals illustrate the gain in weight of the control animals, i.e., about 3.25 grams per day. Animals fed the reduced amount of ascorbic acid (Group IIIa) gained only 1.5 grams per

day. Group IIIb, pair-fed to the latter group but on a normal ascorbic acid intake, gained less than the controls but more than those on the deficient diet.

Group IV guinea pigs gained weight at a rate comparable to Group IIIa animals during the 48 days they were on the diet containing only 0.4 mg. ascorbic acid per day, but gained weight rapidly during the last 27 days while on a normal diet and, at sacrifice, had almost caught up to the controls.

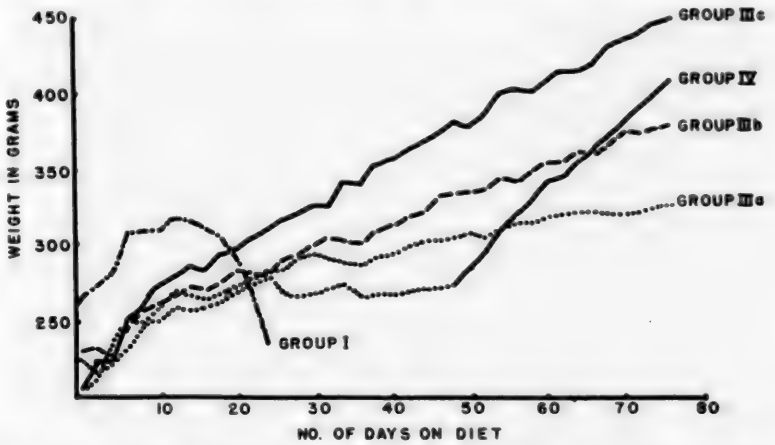

Fig. 1.—A graph showing the weight records of groups of guinea pigs while fed diets containing various amounts of ascorbic acid for various periods of time (see Table I).

Microscopic Observations.—

All of the tissues in the control animals, i.e., Groups IIc and IIIc, appeared normal microscopically. Both groups on the optimal level of dietary ascorbic acid but pair-fed to those on a deficient diet (Groups IIb and IIIb) also appeared normal microscopically. Thus, the reduced food intake of these animals did not appear to have had any effect on their dental tissues.

In all other animals, i.e., those on a daily intake of 0.4 mg. ascorbic acid and those on a diet completely deficient in the element, there were tissue alterations due to the deficiency. The changes were most severe in Group I, in which, for example, areas of hemorrhage (not evident in other groups) occurred in the pulp and periodontal membrane. Certain of these changes are described in detail below.

In the animals receiving no ascorbic acid, there were very severe disturbances in the number and arrangement of the collagen fiber bundles in the periodontal membrane. Fibers were virtually absent in the region just occlusal to the enamel organ and above the crest of the interdental bone septum, i.e., in areas where collagen bundles normally form (Figs. 2 and 3). Fiber bundles, which had developed above the crest of the interdental septum before the animals' reserve of ascorbic acid had been exhausted, had moved occlusally and could still be seen in the interdental region just below the oral epithelium.

The cement pearls, which normally cement the periodontal fibers to the enamel surface, were much smaller than normal, and they developed at a higher level on the tooth than they did in control animals. (The pearls visible in Fig. 2 [right] were formed before the ascorbic acid reserve was depleted in the animal.) The secondary layer of cementum, which normally covers each pearl and spreads out to each side of it, was also reduced in amount. On the

Fig. 2.—Low-power photomicrographs of longitudinal sections of guinea pig molars, each showing an interdental papilla between two molars. *Left*—Normal. *Right*—Scorbutic. The tip of the bony interdental septum is below, and the oral epithelium above. Note the lack of definitive fibers and the epithelial proliferation in the right section.

tooth surface where the periodontal fibers attach directly to dentin, it was difficult to determine whether any cementum had formed at all during the period of the depletion.

The dentin which covered the pulpal surface of older (pre-experimental) dentin in the teeth first lost its normal tubular arrangement and finally ceased forming altogether.

Bone resorption varied markedly from the normal patterns as described by Hunt.[10] Extensive bone resorption occurred over the entire surface of the alveolar bone (see Fig. 3 [right], Fig. 5, Fig. 5, *c*) and it appeared to be rapid, judging from the large number and the size of the osteoclasts. This represented a generalized resorption superimposed on the normal bone resorption

pattern seen in the controls. For example, in areas of pressure where resorption normally occurs, the number of osteoclasts was considerably increased in the deficient animals and, in addition, resorption was also evident, although to a lesser degree, in areas where it normally does not occur (Fig. 5, *a* and *c*). Resorption was also increased at the bone margins directly below the apices of the teeth, and since no deposition of new bone occurred at the lower border of the jaw, the mandible was almost eroded through in this region (Fig. 4). Lack of bone deposition was not unique with the mandibular border, as it was not evident at any site.

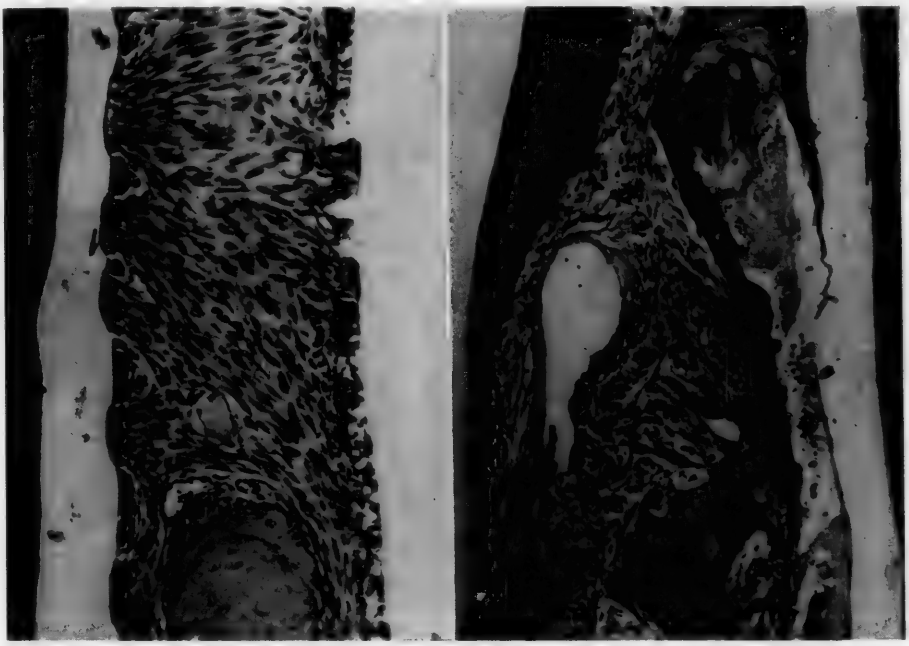

Fig. 3.—High-power photomicrographs of a portion of the interdental papilla between two molar teeth in guinea pigs. *Left*—normal. *Right*—Scorbutic. Note the relative lack of fibers above the bone crest in the section from the scorbutic animal, and the epithelial proliferation in this area. Osteoclasts, indications of bone resorption, are visible in the scorbutic animal.

In the enamel organ also, there were changes brought about by the ascorbic acid deficiency. At the base of the teeth, ameloblasts differentiated, although the enamel organ was much folded (Fig. 4). Enamel matrix was produced in areas where predentin formed, but not in areas where predentin was missing. Alterations were also observed in the residual cells of the enamel organ (reduced ameloblasts and stratum intermedium) further up the tooth. Stratum intermedium cells began to proliferate at a level below the crest of the interdental septum, much closer to the tooth apex than normal (Figs. 2 and 3). At this site, a layer of epithelial cells, derived from the stratum intermedium, spread over and around the cement pearls. In some cases, a continuous layer of cells, several cells thick, covered all the pearls in the area and, in other cases,

isolated islands of cells, also several layers in depth, were interspersed between the cement pearls (Fig. 2). In the most bizarre specimens, the residual stratum intermedium cells proliferated and differentiated to the point where a keratin layer was formed against the tooth surface (Fig. 3).

It is of significance that cellular proliferation, observed at this low level on the teeth, did not result from the apical migration of cells of the oral epithelium, but rather from the multiplication of cells already present in the area. Extension of oral epithelium cells did take place, but only along axial tooth surfaces not covered with enamel.

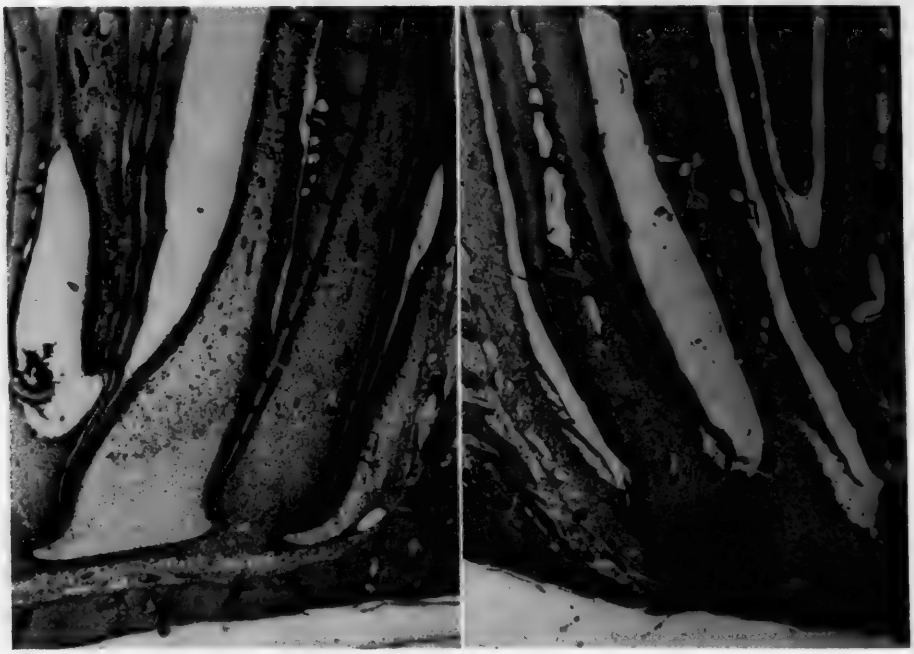

Fig. 4.—Low-power photomicrographs of a portion of the mesial side of the second molar teeth in two guinea pigs. *Left*—Normal. *Right*—Scorbutic. Note the irregular enamel organ and the thinness of bone below the tooth in the scorbutic animal.

No vertical change in the location of the base of the gingival crevice was seen in the interproximal areas which were protected, but pocket formation was seen in all unprotected areas.

In all animals which were fed a diet containing 0.4 mg. ascorbic acid per day (IIa and IIIa), all the tissues were normal, with two exceptions—dentin formed during the period of the deficiency appeared highly disorganized, a derangement similar to that described for ascorbic acid deficiencies elsewhere (Fish and Harris[7]), and evidence of bone resorption was seen at the lingual alveolar bone margin of the lower jaw where it normally does not occur (Fig. 5, *b*). This resorption was neither extensive nor rapid, since only a few small osteoclasts were visible. It was evident in only 5 of

the 7 animals which had been on the deficiency for 75 days and was not seen in those on the deficiency for only 48 days; otherwise bone formation appeared normal in all the animals receiving 0.4 mg. ascorbic acid per day.

In the Group IV animals which were given 0.4 mg. ascorbic acid per day for 48 days, and then 5.0 mg. per day for an additional 27 days, all the tissues being produced at the time of sacrifice were normal histologically. The only evidence that these animals had suffered from a deficiency was the presence of residual abnormal dentin in the teeth located some distance from the pulp, and which had not yet worn off at the occlusal surface.

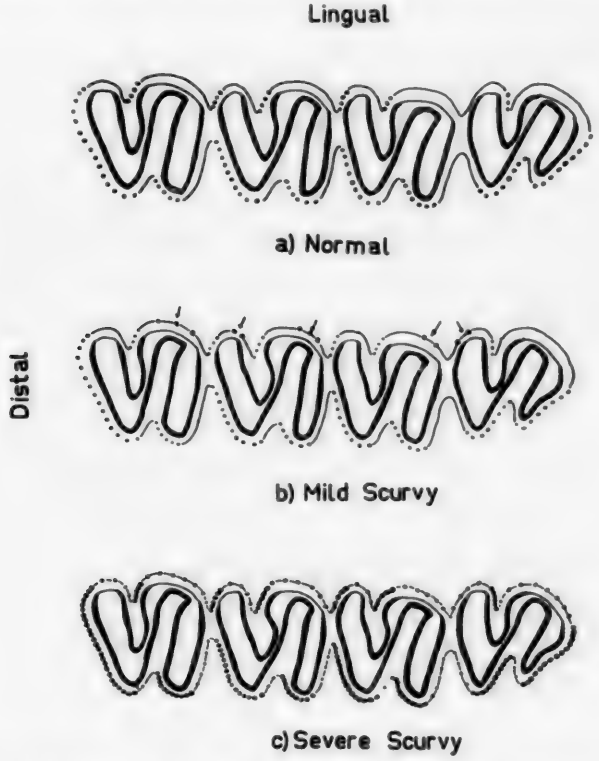

Fig. 5.—Diagrams of cross sections of the mandibular molar teeth of guinea pigs to show resorption of bone around the teeth caused by different degrees of scurvy. The broken line around the teeth indicates the walls of the alveoli, and the dots indicate bone resorption. (*a*) Normal, (*b*) mild scurvy, (*c*) severe scurvy.

DISCUSSION

Lloyd and Sinclair[11] estimated that the average ascorbic acid intake of an adult guinea pig receiving ordinary laboratory rations, supplemented with cabbage ad libitum, is above 25 mg. per day. Mannering[12] reported that, unless the intake falls below 2.0 mg. per day, no microscopic tissue alterations attributable to an ascorbic acid deficiency will result. Therefore, in the present study, it was felt that an allotment of 5.0 mg. ascorbic acid per day per animal

should be sufficient for normal growth and development. The fact that no gross or microscopic abnormalities were observed in any of the groups receiving this amount supports this view.

Mannering[12] further stated that an allowance of 0.4 mg. ascorbic acid per day was just sufficient to support growth in the guinea pig, and that microscopic evidence of scurvy became apparent when the daily ration fell below 0.5 mg. per day. In our study, since the animals receiving 0.4 mg. of the vitamin per day showed microscopic tissue alterations, a hind limb paralysis, and less weight gain than normal, they were assumed to be suffering from scurvy. Because the control pair-fed animals appeared normal grossly and microscopically, although they did not gain the same amount of weight as the ad libitum-fed controls, it seemed evident that the paralysis, the microscopic tissue alterations, and part of the lack of weight gain observed in the animals receiving 0.4 mg. per day, was in fact due to an ascorbic acid deficiency and not simply to inanition.

The guinea pigs on the diet completely deficient in ascorbic acid (Group I) had lost about one quarter of their peak weight (see Fig. 1) and were close to death at the time of sacrifice on the twenty-fourth day of feeding. Their behavior in this respect was very similar to that of the pigs described in the experiment of McLean, Sheppard, and McHenry[1] and typical of severe scurvy.

The connective tissues were most severely affected by the deficiency and, as might be expected, the tissue changes were most severe in the group receiving no dietary ascorbic acid. Alterations were also observed in epithelial tissues, but these were believed to be secondary to those of the connective tissues.

In the guinea pig, there are four sites where collagen fibers continually form in the periodontal tissues in order to provide support for the continually erupting molar teeth. Fibers form in the region just above the enamel organ in the central perpendicular bundle of fibers between the alveolar bone and the enamel surface (Sicher[13] and Santone[14]), and just above the crest of the interdental septum. The latter are carried occlusally as interdental fibers as the teeth erupt (Hunt[10]). Finally, a slower type of fiber replacement and synthesis occurs at the alveolar surface as the bone is remodeled and new fibers are enclosed (Hunt[10]).

In this study, in the jaws of the animals completely deprived of ascorbic acid, there were no collagen fibers present in either the area just above the enamel organ or just above the crest of the interdental septum. Lack of demonstrable fibers in these sites could have resulted from either the breakdown of fibers which had formed or from a lack of synthesis of fibers. Since some thin collagen bundles were observed, both just below the oral epithelium and in the area between the alveolar bone and the enamel surface, but some distance above the enamel organ, it was obvious that if fibers were formed and then broken down, the breakdown was occurring only in selected areas of the periodontal membrane. It is difficult to believe such a selective dissolution occurred.

On the other hand, since the interdental fibers which form just above the crest of the septum are carried occlusally as the teeth erupt, it might be assumed that those visible just below the oral epithelium in the acutely deficient animals had formed several days previously in the region of the bone crest, and at a time when some ascorbic acid was still present in the tissues. In terms of the number of days involved, this seems to have been the case. Fish and Harris[7] estimated that tooth material formed at the apex wears off at the occlusal surface of the guinea pig molar in about 40 days. The distance from the crest of the interdental septum to the oral epithelium is about one quarter the total length of the tooth and, hence, fibers formed at the septal crest would be present as interdental fibers for about 10 days before they disappear. Since the weight loss in Group I animals occurred from approximately the fourteenth to the twenty-fourth day on the diet, one could assume that the animals' reserves of ascorbic acid were used up about 10 days before sacrifice. On the basis of this evidence, we believe the assumption to be correct, i.e., that the interdental fibers present just below the oral epithelium in the Group I animals were formed at the bone crest region at a time when some ascorbic acid was still present for fiber synthesis. The same reasoning could be applied to explain the presence of fibers between the alveolus wall and the tooth enamel, some distance above the enamel organ.

Thus, it appears that the defects observed within the periodontal membrane were due to lack of fiber synthesis rather than fiber destruction, although no further light has been shed on the problem as to whether the ascorbic acid deficiency exerts its effect extra- or intracellularly.

In the animals with severe scurvy, the amount of primary and secondary cementum was much less than normal. Whether this reduction occurred because there was no need for cemetum since there were no fibers to be attached to the teeth, or whether the ascorbic acid deficiency prevented cementum synthesis, cannot be determined. The earliest steps in the formation of cement pearls along the enamel were visible, i.e., the openings in the layer of ameloblast cells occurred, but the process stopped at this stage. It seems likely that, since cementum is one of the connective tissues, its production, like that of collagen fibers, was prevented by the lack of available ascorbic acid.

In the teeth of all of the animals suffering from scurvy, whether severe or mild, marked alterations occurred. These were the only abnormalities (except for delayed, moderate changes in the pattern of bone resorption) observed in those with mild scurvy, a fact which confirms the statement of Keuther, Telford, and Roe[15] that the teeth of the guinea pig are the most sensitive indicators of scurvy.

Since Hunt[10] has described the normal pattern of bone deposition and resorption within the jaws of guinea pigs in detail, it is now possible to assess, quite accurately and sensitively, deviations from the normal pattern displayed by animals under various experimental conditions. In the present study, little change was observed in the animals on the diet containing a daily level of ascorbic acid of 0.4 mg. No alterations were seen after only 48 days

on the diet but, after 75 days, resorption was occurring in areas where it normally did not occur. Bone deposition still occurred at this level of dietary ascorbic acid. In severe scurvy, however, bone deposition ceased and a generalized bone resorption occurred throughout the jaws. The resorption was more extensive in areas where pressure normally occurred along the bone surfaces.

Glickman,[2] in describing the epithelial changes occurring around the molars of severely scorbutic guinea pigs, noted the proliferation of epithelium along the enamel surfaces. He interpreted this proliferation as the result of apical migration of oral epithelium along the teeth. However, Hunt[10] has shown that as the tooth erupts, cells of the stratum intermedium of the enamel organ proliferate and are carried up to meet the basal cells of the oral epithelium, with which they fuse. We have concluded from the present study that since collagen fibers did not form within the interdental area, and thus the residual cells of the enamel organ were not confined as they normally are, the stratum intermedium cells proliferated at a much lower level on the tooth than normal. When these fused with the basal cells of the oral epithelium, the illusion was created that the elongated epithelial contact with enamel resulted from a migration of oral epithelium apically.

SUMMARY

Observations have been made of the teeth and jaws of guinea pigs fed a basal semisynthetic diet supplemented with pure ascorbic acid at levels of 0.0, 0.4, and 5.0 mg. per day per animal for various periods of time. Pair-feeding methods were used as a control.

In animals fed 5.0 mg. ascorbic acid per day, whether fed ad libitum or pair-fed to one of the deficient groups, no microscopic alterations were visible in any of the tissues.

In guinea pigs fed a diet containing 0.4 mg. ascorbic acid per day, severe alterations in the structure of dentin were observed, and mild alterations occurred in the normal pattern of bone deposition and resorption in the jaws. The latter changes were not visible after 45 days' feeding but were seen after 75 days' feeding.

Tissue changes caused by feeding 0.4 mg. ascorbic acid per day for 48 days were reversed by feeding 5.0 mg. per day for an additional 27 days.

Animals kept on a diet completely deficient in the vitamin showed the most severe alterations from normal. Areas of hemorrhage occurred in both the pulp and periodontal membrane. There was a failure of connective tissue formation of collagen fibers, cementum, dentin, and bone. Excessive resorption of bone occurred and was greatest in areas where pressure normally occurs on the bone.

Enamel matrix failed to form in areas where predentin did not form.

Cells of the stratum intermedium of the enamel organ proliferated at a level on the tooth far apical to that at which such proliferation normally occurs. In some areas, a cornified layer was formed. This proliferation of

stratum intermedium cells created the illusion that cells of the oral epithelium had migrated down the side of the tooth.

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BLOOD FLOW AND BLOOD PRESSURE IN THE MANDIBULAR ARTERY

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THE health and resistance of all mammalian tissue are dependent upon an adequate blood flow. Young[1] has called attention to the fact that adequate circulation is necessary for the health of periodontal tissue. In particular, he states that edema of the gingiva resulting from venous stasis is a possible cause of periodontal diseases. Brewer, Muhler, and Fisher[2] have ligated the mandibular arteries of rats. Their experiments show an increase in the number of carious lesions and an increase in the extent of existing lesions after ligation. Glickman and his co-workers[3] determined the oxygen consumption in normal and inflamed human gingiva using the Warburg technic. Since the principal function of the cardiovascular system is to meet tissue demands for transportation of oxygen, carbon dioxide, and nutrients, it is highly important to determine the normal blood flow and blood pressure to these oral tissues. This provides a basis for the further study of those variations which might occur in pathologic states exhibited by oral tissues.

EXPERIMENTAL PROCEDURE

Unilateral blood flow and blood pressure measurements were made in the mandibular artery of mongrel dogs by means of direct cannulation at the level of the mandibular foramen. A minimum of 6 animals were used for the study of each variable. Weights of the animals ranged from 12 to 26 Kg., and anesthetization was induced with sodium pentobarbital, 33 mg./Kg. of body weight given intravenously. Heparin (5 mg./Kg.) was used as an anticoagulant. After administration of these agents, at least 30 minutes elapsed before assays were initiated, in order to minimize the effect of these agents on the vascular system. The rate of volume blood flow was measured by use of an electromagnetic blood-flow meter similar to that described by Richardson, Denison, and Green[4]; flow was recorded on a 5-milliampere Esterline-Angus recorder. This instrument features a linear reproduction of both forward and back flow transients within 5 per cent error. Blood pressure was recorded simultaneously by use of a 22-gauge needle inserted into the lumen of the proximal tubing connecting the mandibular artery to the cannula.

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The needle was attached to a P-23 AA Statham strain gauge filled with heparinized normal saline solution, and a Brush strain analyzer; and blood pressure was recorded on an ink-writing oscillograph. Peripheral vascular tone was calculated in peripheral resistance units, PRU, as described by Green.[5]

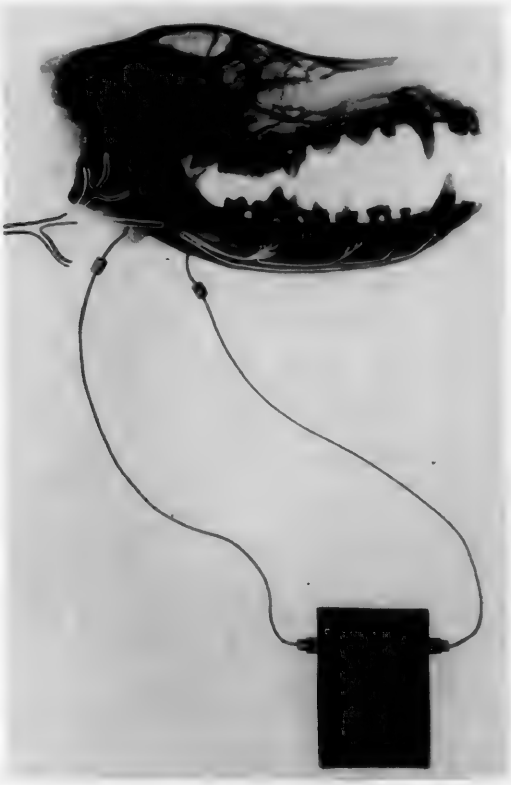

Fig. 1.—Diagrammatic illustration of polyethylene cannula position fixed in the electromagnetic field.

The mandibular artery was exposed by an inframandibular incision. Cannulation was accomplished by insertion of polyethylene tubes with a bore size comparable to that of the lumen of the artery. Fig. 1 diagrammatically illustrates the extent of the vascular bed involved and the site of cannulation. The extent of the vascular bed was determined by injecting Thorotrast directly into the cannula with simultaneous radiographic visualization.

Blood pressure instrumentation was calibrated prior to studies on each experimental animal. The electromagnetic blood-flow meter was also calibrated during measurements on each animal. Flow calibration technic required interruption of the distal side of the cannula and collection of the blood in graduated vessels for 15-second intervals. A calibration curve was

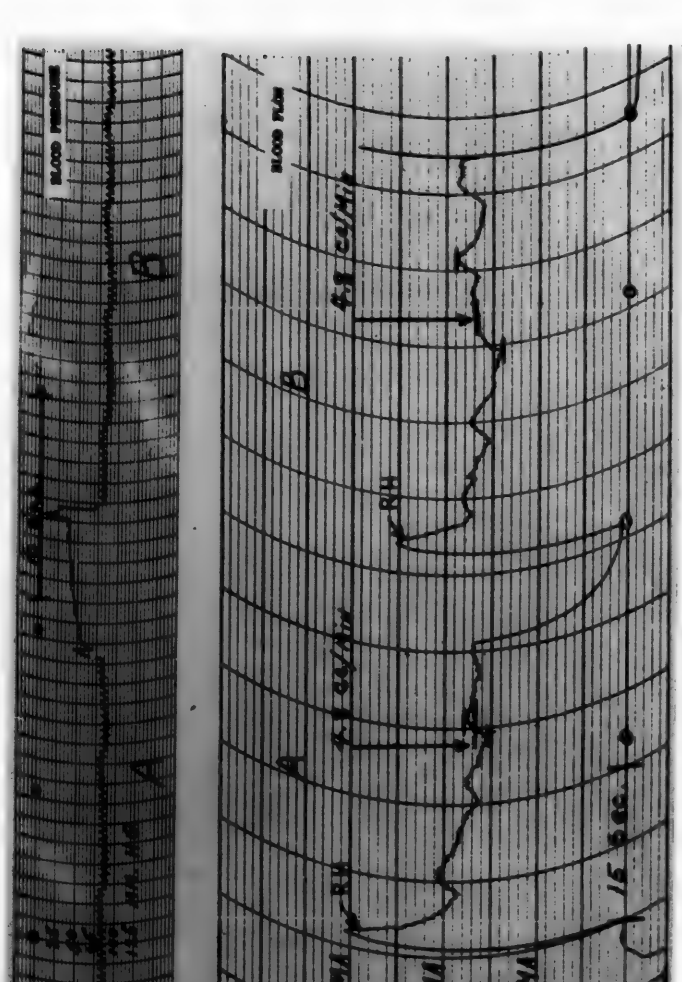

Fig. 2.—Typical blood pressure (upper) and blood flow (lower) recordings. Mechanical zero for blood flow was established by clamping off the polyethylene tube proximal to the cannula. Reactive hyperemia (RH) follows interrupted flow. Mean flow was calculated from the maximum and minimum excursions resulting from the respiratory waves.

obtained by plotting milliamperes deflection on the ordinate against determined volume flows on the abscissa. Flow was mechanically interrupted at intervals, throughout the experiment, to check instrument drift at zero flow. This procedure was considered essential due to the high gain required to measure small volume flow.

Effective doses of epinephrine and acetylcholine, in volumes of 0.1 ml., were infused directly into the distal side of the cannula. As a result, the peripheral vasomotor changes could be delineated from the central effect. Further evaluation of the responsiveness of the vascular bed was made by stimulating the exposed mandibular nerve with 10 volts AC for periods of 1 to 2 seconds.

RESULTS

Determinations of blood flow and blood pressure were made on 22 dogs. Fig. 2 presents typical records of blood pressure (Brush recorder) and blood flow (Esterline-Angus) measurements. Systolic and diastolic blood pressures were recorded in milligrams of mercury with each small division of the ordinate representing 5 mm. Hg pressure. Time was indicated on the abscissa. Zero blood flow was monitored by occlusion of the polyethylene tubing, proximal to the pressure transducer, a procedure which resulted in simultaneous decreased blood pressure recordings. Interruption of blood flow induced tissue ischemia, which was followed by reactive hyperemia. Blood flow was recorded as milliamperes deflection on the ordinate, and time on the abscissa.

The effect of epinephrine, 0.1 μg, injected directly into the distal side of the cannula is demonstrated by Fig. 3. Vasoconstriction is indicated by the increased blood pressure and decreased blood flow. The vasodilating effect of acetylcholine, 1.0 μg, injected distal to the cannula is illustrated in Fig. 4. Fig. 5 shows clearly the abrupt decrease in blood flow resulting from stimulation of the mandibular nerve with 10 volts AC, for a period of 1 to 2 seconds.

Table I presents the mean blood flow and pressure for 22 animals and the data showing the response of flow and pressure to epinephrine, acetylcholine, and nerve stimulation. Mean blood flow for 22 animals was 5.95 c.c. per minute, with the standard error of the mean $\pm$0.57. Mean blood pressure was 105 mm. Hg. The standard error of the mean was $\pm$4.18. Mean peripheral resistance was 20.6 units with the standard error of the mean $\pm$2.02. The injection of 0.1 μg of epinephrine resulted in an average decrease in blood flow from 5.95 c.c. per minute to 2.4 c.c. per minute, representing a decrease of 60 per cent in blood flow. The mean peripheral resistance increased from the control level of 20.6 to 62.6 which represents a 200 per cent increase in the resistance of the vascular bed. Vasodilation was demonstrated with acetylcholine, 1.0 μg, and an average increase of 34.5 per cent in blood flow and a 33 per cent decrease in peripheral resistance was recorded.

Stimulation of the mandibular nerve produced a 70 per cent reduction in blood flow from the control values, within 1 to 2 seconds following stimulation, and effected a 226 per cent increase in peripheral resistance.

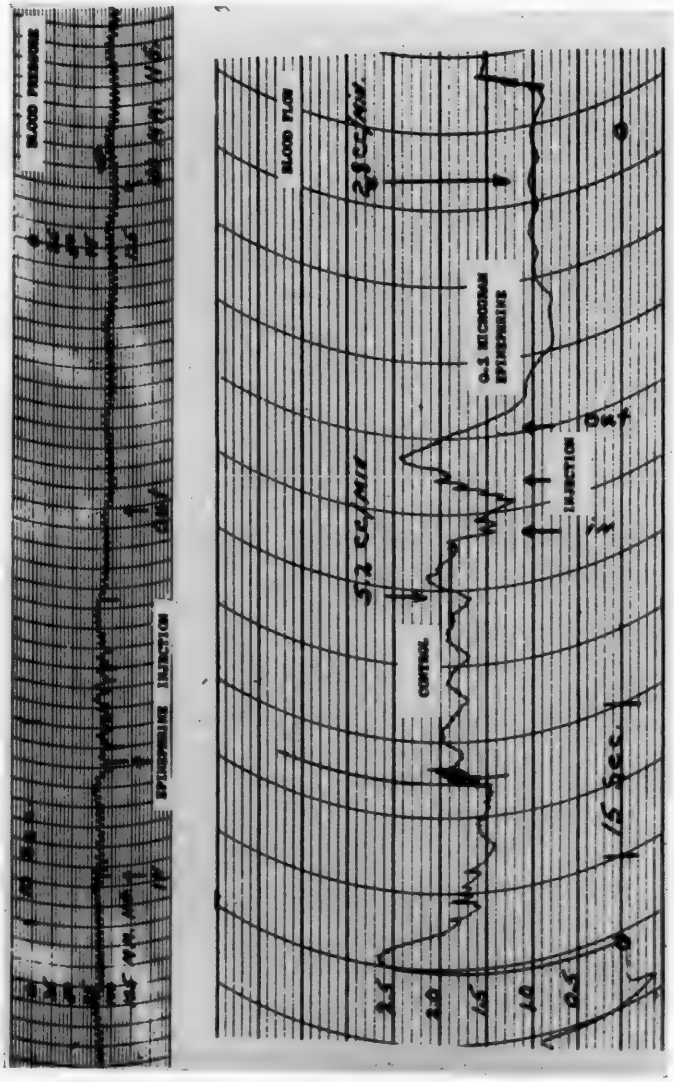

FIG. 3.—Cardiovascular responses to 0.1 μg epinephrine. Control blood flow and blood pressure records precede epinephrine injection. Respiratory waves are characteristically present at the control periods and are diminished by vasoconstriction.

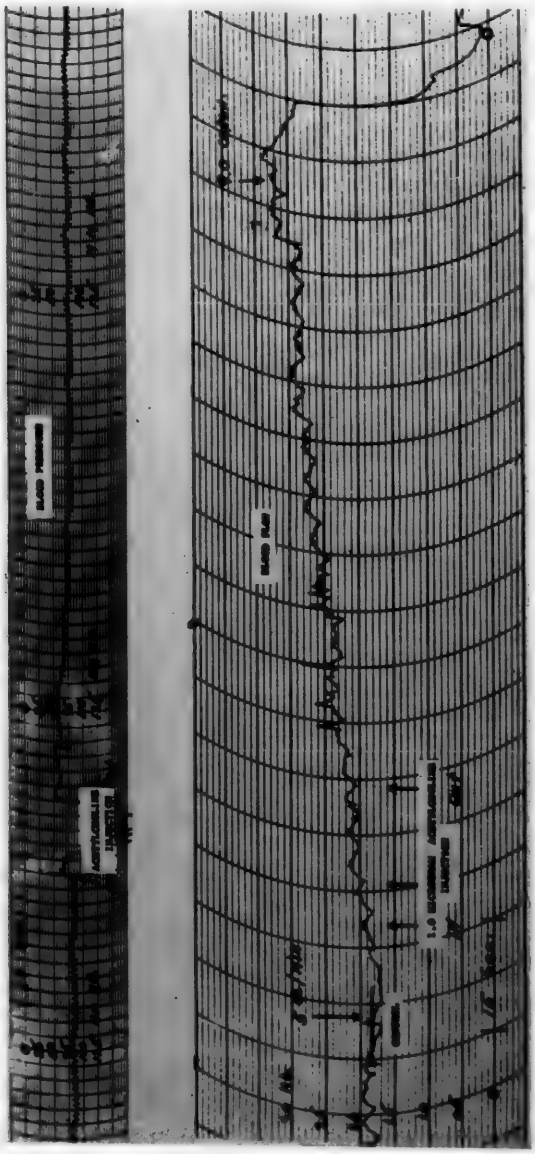

Fig. 4.—Cardiovascular responses to 1.0 μg acetylcholine, preceded by control pressure and flow.

TABLE I
MEAN BLOOD FLOW AND BLOOD PRESSURE DETERMINATIONS FOR 22 EXPERIMENTAL ANIMALS*

	ANIMAL WEIGHT (KG.)	NUMBER OF ANIMALS	NUMBER OF RECORDINGS	BLOOD FLOW (C.C./MIN.)	BLOOD PRESSURE (MM. Hg)	MEAN PERIPHERAL RESISTANCE
Mean control values	18.5 (±0.89)	22	22	5.95 (±0.57)	105 (±4.18)	20.6 (±2.02)
10 seconds after epinephrine	18.6 (±1.3)	9	9	2.4 (±0.36)	130 (±7.4)	62.6 (±12.5)
10 seconds after acetylcholine	19.9 (±1.2)	9	9	8.0 (±1.3)	91 (±10.9)	13.7 (±2.1)
Mandibular nerve stimulation	19.4 (±1.5)	6	6	1.8 (±0.24)	98 (±8.6)	67.1 (±16.2)

Numbers in parentheses show standard error of the mean.

*Peripheral resistance was calculated for each animal and the mean PRU determined from these data. These values served as controls in order to evaluate the effects of epinephrine, acetylcholine, and mandibular nerve stimulation.

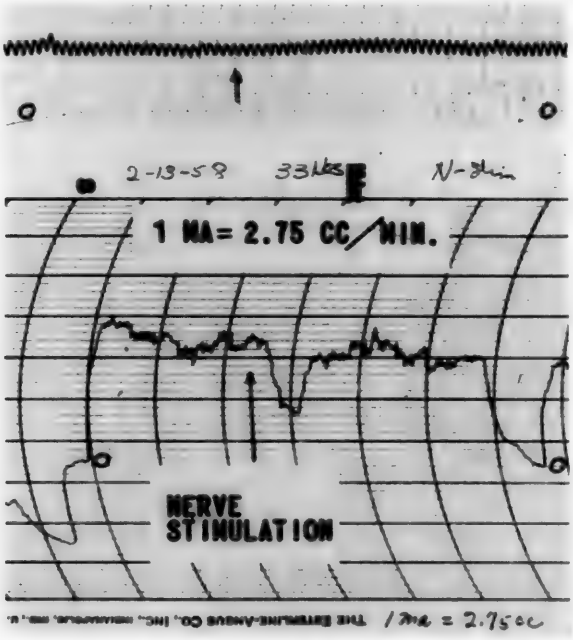

Fig. 5.—Cardiovascular responses to stimulation of the mandibular nerve with 10 volts AC.

DISCUSSION

The determinations of blood flow, blood pressure, and peripheral resistance in the mandibular artery provides an experimental basis for the evaluation of the effects of therapeutic agents, dental operative procedures, and oral pathologic syndromes on the vascularity of oral tissue. Peripheral resistance values obtained suggest a higher vascular tone than that indicated by investigators working with other tissues.[6] The administration of effective doses of epinephrine produced striking changes in the resistance of the vascular bed. In a few animals, flows below 0.5 c.c. per minute were obtained

following the administration of 0.1 μg of epinephrine. Although the vasomotor tone of the bed appeared to be somewhat higher than in other tissues, it was still capable of considerable further increase. The vascular bed of the mandible was considered an area of high vasomotor vascular tone.

Further evidence for evaluating the responsiveness of the vascular bed was obtained by injecting acetylcholine and by stimulating the mandibular nerve. Acetylcholine, 1.0 μg, did not significantly decrease blood pressure from control values, although peripheral resistance was significantly decreased. Vasodilatation probably could have been accentuated by increasing the amount injected, since the same dosage in smaller animals usually produced greater changes. Stimulation of the mandibular nerve resulted in a significant vasoconstriction, indicating the presence of vasoconstrictor fibers in the nerve.

Variations in the magnitude of blood pressure and blood flow were demonstrated in the 22 animals. The use of mongrel dogs of mixed breeds, age, sex, and body weight was undoubtedly partially responsible for the variations obtained from the group. Other factors, such as plane of anesthesia and pain, are known to affect blood pressure. Although blood flow may be influenced by these variables, the metabolic demands of the tissue involved may also be a factor. Intermittent interruption of flow produced by clamping off the distal side of the cannula was followed by a transient rise in flow, which returned quickly to a level close to that of the control value. This transient rise in flow is interpreted as representing vasodilation resulting from temporary tissue ischemia and suggests the presence of local regulation of the vascular bed in this region. It would be interesting to determine if the PRU levels in animals with oral disease are demonstrably different from those recorded in this study.

The determination of blood flow and blood pressure on one side of the mandible is dependent upon the assumption that minimal crossover of flow occurs between the right and left side of the mandible. This apparent unilateral blood supply indicates that the dog is a suitable animal for investigators using this technic.

SUMMARY

1. Blood flow and blood pressure were measured in the mandibular artery in 22 mongrel dogs.

2. Peripheral resistance of the vascular bed of the mandible was high (20.6).

3. Acetylcholine produced a limited vasodilation of the vascular bed in doses of 1.0 μg.

4. Epinephrine produced marked vasoconstriction effects on the artery in doses of 0.1 μg.

5. Stimulation of the mandibular nerve produced vasoconstriction comparable to that elicited by epinephrine administration.

The authors wish to express their gratitude to G. Cameron, C. Puckett, and T. Clem for their technical assistance in this project.

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CALCIFICATION

XXII. A Method of Studying Crystal Growth

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STUDIES of in vitro calcification indicate that once nuclei of crystallization are formed in preosseous cartilage, further crystal growth is independent of the nucleus-forming mechanism. Thus, the degree of calcification detected by the silver nitrate stain represents the gross effect of two discrete processes which are not always distinguishable. For example, complete inhibition of in vitro calcification after an experimental treatment may be due not only to an injury of the nucleus-forming mechanism but also interference with the growth of nuclei which are below the threshold of visibility as determined by the silver stain. The purpose of this paper is to describe a method for the study of crystal growth independent of the nuclei-forming mechanism.

This method depends upon the seeding of rachitic or demineralized tibia sections by a 2-stage calcium and phosphate treatment. The tibia sections then act as a collector of further mineralization and may be readily isolated for qualitative examination by the silver stain, or quantitative estimation by chemical analysis. It is also possible that nuclei produced by this method possess properties such as particle size and orientation similar to nuclei produced in vivo. This method is derived from the early experiments of Freudenberg and György,[1] who found that calcium uptake in cartilage is a prerequisite for the uptake of phosphate in cartilage. Boyd and Neuman[2] and Belanger[3] have corroborated this work and related the calcium uptake to the mucopolysaccharide content of cartilage.

MATERIALS AND METHODS

The bones used were the tibia of Wistar strain rats made rachitic, as previously described,[4] by the use of a high-calcium, low-phosphate diet.

Decalcification was carried out by placing tibia sections in a 3 per cent solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA) for 48 hours at a pH of 4. This was followed by washing with distilled water.

A stock calcium chloride solution containing 250 mM/L. was prepared by weighing out 25.023 grams of calcium carbonate, which was converted to the chloride by means of hydrochloric acid. All excess hydrochloric acid was removed by drying at 110° C., and the final solution filtered.

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A stock phosphate solution containing 333 mM/L. was prepared by weighing out 35.5 grams of Na_2HPO_4 and 11.5 grams of $Na_2HPO_4 \cdot H_2O$ per liter. The pH of this solution was 7.2 which rose to 7.3 upon dilution to 50 mM/L.

The method of in vitro calcification has been previously described.[4] The basal salt solution contains 0.07 M NaCl, 0.005 M KCl, and 0.022 M $NaHCO_3$ per liter in addition to the calcium and phosphate used in a specific experiment.

Prior to calcium and phosphate analysis,[5] bone samples were soaked overnight in absolute alcohol, then in absolute ether, and in alcohol again and dried overnight at 105° C. The validity of the analysis was checked by the use of powered cattle bone of known calcium and phosphate content.

The degree of calcification was determined by adding 3 per cent silver nitrate, illuminating with a 60 watt bulb for 5 minutes, and grading in accordance with a well-established procedure[6] from 0 (0) to 4 (++++). The width of the calcified area is designated by the number preceding the parentheses, and its length by the (+) number.

After the usual conditions of in vitro calcification, rachitic tibial slices usually show a zone of noncalcified cartilage above the calcified area. This is referred to as "nonossifiable" cartilage and corresponds to the narrow band of uncalcified cartilage of young, normal rats on a nonrachitogenic diet. When the use of nonphysiologic concentrations of calcium and phosphate resulted in calcification in this zone, it is noted by the letter N following the degree of calcification (i.e., 4 (++++) N).

EXPERIMENTAL FINDINGS

A. Rachitic sections, decalcified by the method described above, were shaken in 75 mM/L. $CaCl_2$ for 1 hour, followed by shaking with various phosphate solutions, varying from 0 to 50 mM/L. for ½ hour. These sections were then incubated in calcifying solution for 18 hours at a Ca (10 mg. %) × P (5 mg. %) = 50. As seen in Table I, mineralization of preosseous cartilage including the nonossifying zone was found to initially occur at 16.7 mM/L. phosphate and was extensive at 50 mM/L. phosphate.

B. Rachitic tibia sections were shaken for 1 hour with 75 mM/L. $CaCl_2$ followed by ½ hour of shaking with 50 mM/L. phosphate, after which the sections were incubated in calcifying solution for 18 hours at a Ca (10 mg. %) : P (5 mg. %) = 50. As seen in Table II, this treatment resulted in mineralization of the entire cartilage, including the nonossifiable zone. One hour of shaking with either distilled water or 75 mM/L. $CaCl_2$, following the original pretreatment, did not interfere with the high degree of mineralization.

C. Demineralized rachitic tibia sections were shaken with 75 mM/L. $CaCl_2$ for 1 hour, followed by shaking with 50 mM/L. phosphate for ½ hour. This was followed by in vitro calcification at a Ca (10 mg. %) × P (5 mg. %) = 50 product for 18 hours. Variations of this pretreatment, such as increasing the calcium chloride concentration to 250 mM/L. and adjusting the pH of the phosphate solution to 9.0, instead of the usual 7.3, were also carried out. The

results of analysis for calcium and phosphate of fresh untreated, decalcified, calcium- and phosphate-treated, and incubated recalcified sections are seen in Table III. Decalcified sections after initial calcium and phosphate treatment were found to have a low mineral level, which was readily distinguishable from recalcified sections in all cases.

TABLE I

IN VITRO CALCIFICATION OF DECALCIFIED BONE SLICES SHAKEN FOR 1 HOUR WITH 75 mM/L. $CaCl_2$ FOLLOWED BY SHAKING FOR ½ HOUR WITH VARIOUS CONCENTRATIONS OF PHOSPHATE (INCUBATION AT Ca (10 MG. %) × P (5 MG. %) = 50 AT pH 7.3 FOR 18 HOURS)

TREATMENT BEFORE CALCIFICATION	DEGREE OF CALCIFICATION*
$CaCl_2$	0 (0)
$CaCl_2$, 8.3 mM/L. phosphate	0 (0)
$CaCl_2$, 16.7 mM/L. phosphate	1.8 (++++) N†
$CaCl_2$, 25 mM/L. phosphate	2 (++++) N†
$CaCl_2$, 33.3 mM/L. phosphate	2.8 (++++) N‡
$CaCl_2$, 50 mM/L. phosphate	3.3 (++++) N§

*The degree of calcification is indicated as follows: 0 (0) no calcification; 1 (+) trace; 1 (++) broken thin line; 1 (+++) almost complete thin line across the provisional zone; 1 (++++) complete thin line across the provisional zone; 2 (++++) heavy line across the provisional zone including the primary tongues of cartilage; 3 (++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (++++) practically complete calcification of the metaphysis.

†Some calcification in "nonossifying" cartilage.

‡Calcification in "nonossifying" cartilage almost complete.

§Calcification in "nonossifying" cartilage complete.

TABLE II

INSOLUBILITY IN WATER AND $CaCl_2$ SOLUTION OF CALCIUM PHOSPHATE NUCLEI FORMED ON FRESH BONE SLICES—NUCLEI FORMED BY TREATMENT WITH 75 mM/L. $CaCl_2$ FOR 1 HOUR, THEN 50 mM/L. PHOSPHATE FOR ½ HOUR (INCUBATION AT Ca (10 MG. %) × P (5 MG. %) = 50 AT pH 7.3 FOR 18 HOURS)

TREATMENT BEFORE CALCIFICATION	DEGREE OF CALCIFICATION*
None	2 (++++)
$CaCl_2$	2 (++++)
$CaCl_2$, phosphate	4 (++++) N†
$CaCl_2$, phosphate, 1 hr. shaking with water	4 (++++) N†
$CaCl_2$, phosphate, 1 hr. shaking with 75 mM/L. $CaCl_2$	4 (++++) N†

*The degree of calcification is indicated as follows: 0 (0) no calcification; 1 (+) trace; 1 (++) broken thin line; 1 (+++) almost complete thin line across the provisional zone; 2 (++++) heavy line across the provisional zone including the primary tongues of cartilage; 3 (++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (++++) practically complete calcification of the metaphysis.

†N refers to calcification in "nonossifying" cartilage.

D. Rachitic sections were shaken with 75 mM/L. $CaCl_2$ for 1 hour, followed by shaking with either 10 mM or 16.7 mM/L. phosphate for ½ hour. This was followed by incubation in calcifying solution at several products, including one of Ca (6 mg. %) × P (3 mg. %) = 18, where in vitro calcification was found to proceed (Table IV). At this product, in vitro calcification does not ordinarily occur.

E. Rachitic sections shaken for 1 hour in 75 mM/L. $CaCl_2$, followed by shaking for ½ hour in 50 mM/L. phosphate, were then shaken with 0.001 M beryllium in water or 0.001 M beryllium in 75 mM/L. in calcium chloride. This was followed by incubation in calcifying solution at a Ca (10 mg. %) : P (5 mg. %) = 50 product. As seen in Table V, this treatment resulted in complete inhibition of calcification. Additional treatment with 50 mM/L. phosphate,

TABLE III
CALCIUM AND PHOSPHORUS IN RACHITIC, DECALCIFIED, CALCIUM- AND PHOSPHATE-TREATED, AND RECALCIFIED TIBIAL SLICES
(RECALCIFICATION BY INCUBATION IN Ca (10 MG. %) × P (5 MG. %) AT pH 7.3 FOR 18 HOURS)

		PER CENT CALCIUM	PER CENT PHOSPHORUS	Ca/P
A	Untreated	7.29	3.40	2.14
	Decalcified	0.00	0.27	0.00
	Ca_3PO_4 treated	0.22	0.73	0.30
	Recalcified	2.41	1.36	1.77
B	Untreated	7.39	3.59	2.06
	Decalcified	0.00	0.22	0.00
	Ca_3PO_4 treated	0.16	0.69	0.23
	Recalcified	2.50	1.38	1.81
C	Untreated	7.21	3.03	2.38
	Decalcified	0.00	0.17	0.00
	Ca_3PO_4 treated	0.28	0.79	0.35
	Recalcified	3.73	1.72	2.17
D	Untreated	6.91	2.81	2.46
	Decalcified	0.00	0.20	0.00
	Ca_3PO_4 treated	0.94	0.56	1.14
	Recalcified	2.21	1.15	1.92
E	Untreated	6.73	3.03	2.22
	Decalcified	0.00	0.20	0.00
	Ca_3PO_4 treated	0.53	0.89	0.60
	Recalcified	3.30	1.69	1.95
F	Untreated	8.12	3.99	2.06
	Decalcified	0.00	0.20	0.00
	Ca_3PO_4 treated	0.42	0.85	0.49
	Recalcified	2.08	1.20	1.73
G	Untreated	5.39	2.71	1.98
	Decalcified	0.00	0.15	0.00
	Ca_3PO_4 treated	0.37	0.78	0.47
	Recalcified	2.01	1.17	1.72
H	Untreated	13.30	5.43	2.44
	Decalcified	0.00	0.21	0.00
	Ca_3PO_4 treated	0.43	0.74	0.58
	Recalcified	2.59	1.62	1.60
I	Untreated	11.69	4.99	2.33
	Decalcified	0.00	0.27	0.00
	Ca_3PO_4 treated	1.41	1.36	1.04
	Recalcified	5.84	2.55	2.29
J	Untreated	9.92	4.39	2.26
	Decalcified	0.00	0.34	0.00
	Ca_3PO_4 treated	1.91	1.59	1.20
	Recalcified	6.04	2.74	2.20

A-D treated 1 hr. with 75 mM/L. $CaCl_2$, and ½ hr. with 50 mM/L. phosphate, pH 7.3.
E-H treated 1 hr. with 75 mM/L. $CaCl_2$, and ½ hr. with 50 mM/L. phosphate, pH 9.0.
I treated 1 hr. with 250 mM/L. $CaCl_2$, and ½ hr. with 50 mM/L. phosphate, pH 7.3.
J treated 1 hr. with 250 mM/L. $CaCl_2$, and ½ hr. with 50 mM/L. phosphate, pH 9.0.

following the original beryllium shaking, was found to reverse only the beryllium in sections treated with beryllium in the presence of calcium chloride.

F. A reagent grade commercial source of $Ca_3(PO_4)_2$ was substituted for the calcium- and phosphate-treated rachitic sections in experiments similar to those described above in E. Ten milligrams of calcium phosphate was shaken

TABLE IV

EFFECTIVENESS OF CALCIFYING SOLUTIONS WITH LOW ION PRODUCT IN CAUSING MINERALIZATION OF TIBIAL SLICES SHAKEN FOR 1 HR. WITH 75 mM/L. $CaCl_2$ FOLLOWED BY SHAKING FOR ½ HR. WITH PHOSPHATE*

(INCUBATION AT pH 7.3 FOR 18 HOURS)

CALCIFYING SOLUTION USED	DEGREE OF CALCIFICATION†	
	PHOSPHATE 10 mM/L.	PHOSPHATE 16.7 mM/L.
Ca (6 mg. %) × P (3 mg. %) = 18	2 (++++) N‡	4 (++++) N‡
Ca (8 mg. %) × P (4 mg. %) = 32	4 (++++) N‡	4 (++++) N‡
Ca (10 mg. %) × P (5 mg. %) = 50	4 (++++) N‡	4 (++++) N‡

*Ordinarily 6 × 3 solution produces no calcification, and 8 × 4 solution much less calcification than 10 × 5. The silver stain was not as intense as usual in the calcification produced at the lower products.

†The degree of calcification is indicated as follows: 0 (0) no calcification; 1 (+) trace; 1 (++) broken thin line; 1 (+++) almost complete thin line across the provisional zone; 2 (++++) heavy line across the provisional zone including the primary tongues of cartilage; 3 (++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (++++) practically complete calcification of the metaphysis.

‡N refers to calcification in the "nonossifying" cartilage.

TABLE V

REVERSIBLE INHIBITION OF CRYSTAL GROWTH IN "NUCLEATED" BONE CARTILAGE*

(INCUBATION AT Ca (10 MG. %) × P (5 MG. %) = 50 AT pH 7.3 FOR 18 HOURS)

TREATMENT BEFORE CALCIFICATION	DEGREE OF CALCIFICATION†
None	2 (++++)
$CaCl_2$, phosphate	4 (++++) N‡
$CaCl_2$, phosphate, 1 hr. shaking with water	4 (++++) N‡
$CaCl_2$, phosphate, 1 hr. shaking with 75 mM/L. $CaCl_2$	4 (++++) N‡
$CaCl_2$, phosphate, 1 hr. shaking with 1 mM/L. beryllium in water	0 (0)
$CaCl_2$, phosphate, 1 hr. shaking with 1 mM/L. beryllium in 75 mM/L. $CaCl_2$	0 (0)§
$CaCl_2$, phosphate, 1 hr. shaking with 1 mM/L. beryllium in water, ½ hr. shaking with 50 mM/L. phosphate	0 (0)§
$CaCl_2$, phosphate, 1 hr. shaking with 1 mM/L. beryllium in 75 mM/L. $CaCl_2$, ½ hr. shaking with 50 mM/L. phosphate	4 (++++) N‡

*All slices except the first one treated with 75 mM/L. $CaCl_2$ 1 hour and 50 mM/L. phosphate ½ hour.

†The degree of calcification is indicated as follows: 0 (0) no calcification; 1 (+) trace; 1 (++) broken thin line; 1 (+++) almost complete thin line across the provisional zone; 2 (++++) heavy line across the provisional zone including the primary tongues of cartilage; 3 (++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (++++) practically complete calcification of the metaphysis.

‡Refers to calcification, in the "nonossifying" cartilage.

§No genuine calcification, but light yellow stain with $AgNO_3$.

with 100 ml. of 0.1 mM/L. beryllium in water or 0.1 mM/L. beryllium in 75 mM/L. $CaCl_2$ during the pretreatment stage. The solid phase was separated by centrifugation, washed with distilled water, and dried at 105° C. An aliquot of 2.5 mg. was then added to 50 ml. of in vitro calcifying solution Ca (10 mg. %) × P (4.8 mg. %) = 48 adjusted to a pH of 7.3 and incubated for 18 hours. Following incubation, the calcium and phosphorus of the supernatant solution were determined. As seen in Table VI, whereas incubation of control samples of calcium phosphate receiving no treatment or shaken previously with either water or calcium chloride resulted in the removal of calcium and phosphorus from solution, the beryllium-treated specimens did not appreciably alter the composition of the calcifying solution. Additional treatment with 50 mM/L. phosphate

following the original beryllium shaking was found to markedly restore the ability of only the beryllium in $CaCl_2$ treated samples of calcium phosphate to remove calcium and phosphorus from solution.

TABLE VI
REVERSIBLE INHIBITION OF CRYSTAL GROWTH OF TERTIARY CALCIUM PHOSPHATE (INCUBATION AT Ca (10 MG. %) × P (4.8 MG. %) = 48 AT pH 7.3 FOR 18 HOURS)

TREATMENT OF $Ca_3(PO_4)_2$ BEFORE INCUBATING 2.5 MG. IN 50 ML. CALCIFYING SOLUTION*	CONCENTRATION OF CALCIFYING SOLUTION AFTER INCUBATION	
	CALCIUM (MG. %)	PHOSPHORUS (MG. %)
None	6.0	3.0
1 hr. shaking with water	6.7	3.3
1 hr. shaking with 75 mM/L. $CaCl_2$	6.8	3.3
1 hr. shaking with 0.1 mM/L. beryllium in water	10.0	4.8
1 hr. shaking with 0.1 mM/L. beryllium in 75 mM/L. $CaCl_2$	9.8	4.7
1 hr. shaking with 0.1 mM/L. beryllium in water; ½ hr. shaking with 50 mM/L. phosphate	9.8	4.7
1 hr. shaking with 0.1 mM/L. beryllium in 75 mM/L. $CaCl_2$; ½ hr. shaking with 50 mM/L. phosphate	7.6	3.9

*In the preliminary treatment, 10 mg. of $Ca_3(PO_4)_2$ was treated with 100 ml. of the indicated solution(s) followed by washing with distilled water and drying at 105° C.

DISCUSSION

Attempts to characterize the mineralization of preosseous cartilage indicate the existence of three discrete phases, starting with the formation of nuclei of crystallization, continuing with their growth, and ceasing upon attainment of a finite crystal size. Only recently has it been possible to distinguish between the nuclei-forming mechanism and the subsequent growth of crystals. This is based on the finding in preosseous cartilage[7] that once nuclei are formed they will continue to grow, despite such procedures as shaking with distilled water, heat treatment, and presence of magnesium in conjunction with iodoacetate; all of which prevent the nuclei-forming mechanism from functioning. Methods for the production and study of nuclei crystallization generally require either preliminary incubation of preosseous cartilage in calcifying solution for several hours or the precipitation of calcium phosphate compounds in simple inorganic systems. On the basis of this study, preliminary shaking of demineralized rachitic sections for 1 hour with 75 mM/L. $CaCl_2$ followed by shaking for ½ hour with 50 mM/L. phosphate results in nuclei which combine advantages from each system. The seeding operation is performed in an organic matrix which may superimpose properties related to orientation and crystal size. The problem of quantitative studies is simplified by the low base level of calcium and phosphate following this treatment of demineralized bone prior to further study of crystal growth by chemical analysis (Table III). This system may also find application in implantation experiments[8] in which rachitic sections acting as a carrier are imbedded under the skin and permit the ready removal of tissue for examination and analysis of the mineral component. In contrast, it would be a difficult task to follow the fate of fine nuclei imbedded in vivo.

The demineralization step may be omitted and fresh rachitic sections used for the production of nuclei by high-calcium and high-phosphate treatment when it is desired to evaluate the degree of mineralization only by the silver stain. As seen in Table I and Table II, this treatment of either demineralized or fresh tibia sections results in extensive mineralization including the "nonossifying" cartilage after incubation in calcifying solutions.

The mineralization of both preosseous and "nonossifying" cartilage is presumably due to the initial binding of calcium which has been shown to be prerequisite to the uptake of phosphate.[1] Thus, high concentrations of phosphate act as an indicator of sites containing calcium. The response of demineralized sections to treatment with a high concentration of calcium, however, has been found to differ from that of fresh sections. For example, shaking of fresh sections with 75 mM/L. $CaCl_2$ followed by in vitro calcification results in 2 (++++) calcification (Table II) in the preosseous cartilage, whereas similar treatment of demineralized sections results in 0 (0) calcification (Table I). A further difference in the response between fresh and demineralized sections can also be demonstrated if the concentration of the phosphate solution under the conditions of this method is lowered to 16.7 mM/L.; being 1.8 (++++) N for demineralized sections (Table I) and 4 (++++) N for fresh sections (Table IV). It is possible that EDTA treatment causes changes in the chemical structure of the organic matrix.

The nuclei produced by the calcium and phosphate treatment are firmly lodged in the organic matrix and are highly resistant to mechanical removal or solution as seen in Table I where 1 hour of shaking in distilled water is without effect on subsequent calcification. Once these nuclei are established, crystal growth can proceed at a product of Ca (6 mg. %) $\times$ PO_4 (3 mg. %) = 18 which is too low to initiate mineralization in rachitic tibia sections (Table IV).

The application of the silver stain to calcium- and phosphate-treated sections prior to incubation in calcifying solution revealed a light yellow-tinged color in contrast to the usual brown-black color. A yellow color was also obtained by applying the silver stain to such purely inorganic calcium phosphate compounds as tricalcium phosphate. It is evident that the brown-black silver stain is dependent on unknown factors in addition to the presence of phosphate and may reflect changes in the organic matrix that accompany mineralization.

The quantitative studies (Table III) showed that the Ca/P ratio of the calcium- and phosphate-treated sections were as low as 0.23 on a weight basis. The amount of organic phosphate indicated in the analysis of the demineralized sections is not sufficient to explain this low value. If the initial precipitate is high in phosphate, it would be expected to be converted to apatite under the influence of the supernatant calcifying solution.[9]

This method has furnished additional information to help interpret the reversible inhibition of calcification in vitro by beryllium.[4] As seen in Table V, beryllium in either water or calcium chloride blocks the further growth of

nuclei. These treatments are not equivalent since the inhibition of only the sections treated with beryllium in calcium chloride can be reversed by additional phosphate treatment under the conditions of the experiment. Analogous behavior is evident when tertiary calcium phosphate was employed instead of "nucleated" bone sections (Table VI). In this system, a lower concentration of beryllium ions was required in the presence of calcium for the inhibition to be reversed. It is suggested that in both the nucleated cartilage and the inorganic crystals, that calcium competitively limits the binding of beryllium at sites essential for crystal growth and/or influences the type or degree of beryllium binding to the crystal surface. The mechanism by which the inhibition due to treatment with beryllium in calcium chloride is reversed requires further study. Phosphate treatment presumably results in the formation of insoluble beryllium phosphate which is either removed or does not interfere with crystal growth.

These findings are in harmony with the concept that ions foreign to the apatite lattice may block crystal growth.[10] The possibility now exists that the inhibition of fresh non-nucleated sections, previously reported,[4] may be a 2-stage process in which beryllium (1) combines with an active site usually occupied by calcium during the process of mineralization and (2) upon subsequent treatment with calcifying solution is liberated to poison crystal growth.

An attempt has been made to explain the phenomena of crystal growth by the theory of spiral dislocations[11] which states that growth occurs when the deposited planes of atoms are slightly out of phase. According to this theory, beryllium limits growth by causing a continuity of the crystal lattice. Critical concentrations of naturally occurring ions such as citrate which are foreign to the apatite lattice[10] may perform this function in vivo.[12] This would explain the difference in size between enamel and bone crystals. Further exploration with the described method, utilizing both test tube and implantation experiments, may contribute to the understanding of crystal growth in mineralized tissues.

SUMMARY

A method is described for the study of crystal growth, which utilizes fresh and demineralized rachitic tibia sections nucleated by shaking with 75 mM/L. $CaCl_2$ followed by shaking with 50 mM/L. phosphate. Nuclei produced by this method resist destruction, as determined by water shaking, are easily handled for in vitro or implantation experiments, and may be readily isolated for qualitative examination by the silver stain. Decalcified sections show a significant difference in the calcium and phosphate content between the nucleated and recalcified stages and may be examined quantitatively by chemical analysis for calcium and phosphate. It was found that sections nucleated by this method continue crystal growth at a Ca : P product of 16, which is too low to form nuclei of crystallization. The use of this method is illustrated in exploring the reversible inhibition of crystal growth by beryllium.

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A BACTERIOLYTIC AGENT IN SALIVARY GLOBULIN OF CARIES-IMMUNE HUMAN BEINGS

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INTRODUCTION

DENTAL CARIES is a bacterial disease affecting the majority of human beings in this country. Neither the etiologic agents nor the mechanisms of injury production have been exactly determined, although the weight of evidence, in some part circumstantial, has incriminated certain oral acidogenic microorganisms. There are a number of people who, although subsisting on "cariogenic" diets, exhibit complete resistance or immunity to dental caries. In many cases, this resistance cannot be associated with any external environmental conditions and, in these cases, the mechanism of resistance may be assumed to be inherent.

An inherent mechanism of dental caries resistance probably operates by reducing the numbers of, or by modifying the activities of, the bacteria which produce caries. It is also probable that the mechanism functions in the saliva, and its antibacterial properties could be either directly aimed at cariogenic bacteria, or it could operate through a secondary mechanism.

Many investigators have found that of the total salivary flora, lactobacilli appear to be most closely associated with dental caries. Several salivary antibacterial factors active against lactobacilli have been found by other investigators.[1-5] These several factors apparently are not identical. Of these workers, Hill[3] in particular found an association of the presence of his salivary factor with caries resistance.

Salivary factors associated with other bacteria have also been found. Dawson and Blagg[6] found two active principles directed against cholera vibrios, which were apparently not proteins. Wheatcroft[7] has demonstrated salivary antibodies in cases of brucellosis, and Coleman and Appleman[8] found syphilis reagin antibodies in saliva. Lysozyme has been recognized in saliva for many years, but its broad range of activity has not been associated with resistance to any specific disease.

It is evident that antibacterial factors of varying specificity may be found in human saliva. This report is part of a continuing investigation into the salivary mechanisms of dental caries immunity, and is preceded by other reports from this laboratory.[9-11]

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METHODS

The adults who furnished saliva for use in this work were of two groups, caries-immune and caries-susceptible. The caries-immune group has been described recently (Weisenstein and Green[11]). The caries susceptibles were dental students, all of whom had open carious lesions which had appeared in the previous year and who had a salivary lactobacilli count greater than 5,000 per milliliter.

The saliva was paraffin stimulated, collection was begun one hour after a meal, and the lots were pooled. It was held overnight at 7° C. to encourage precipitation of mucin. The heavy flocculent precipitate was removed by glass-fiber filtration, and the remainder was passed through 2 thicknesses of Whatman No. 1 filter paper in a Buchner funnel, using suction. This filtrate was passed through a Selas porcelain bacteriologic filter and was stored in vaccine bottles at –10° C. Sterility was checked by inoculation of samples into thioglycollate broth.

Salivary lactobacilli were classified according to colony type only (Green and Dodd[9]). Tomato juice broth was prepared by Hadley's method, and for use in plates, 2 per cent agar, 0.004 per cent bromcresol green, and 0.0001 per cent sodium azide were added. Rogosa's LBS medium[12] was modified to liquid form by omission of acetic acid, agar, and 5 grams of sodium acetate.

The bacteriolytic effect of saliva was demonstrated in the following manner. Photometer tubes were prepared containing 4 ml. of brain-heart infusion broth (Difco) plus 0.05 per cent yeast extract and 0.5 per cent glucose. The stoppered tubes were sterilized at 15 pounds pressure, 121° C. for 15 minutes. Six milliliters of sterile saliva was added aseptically just prior to inoculation. The inoculum was 0.3 ml. of a saline suspension of 72 hr. lactobacilli adjusted to 0.4 optical density at 610 mμ. The inoculated tubes were observed immediately and at intervals thereafter in a Coleman Junior spectrophotometer at 500 mμ, using distilled water as the blank for 100 per cent light transmission. Uninoculated tubes of media served as controls of the optical changes in the media. Incubation was at 37° C.

Sterile, filtered saliva was adsorbed by adding a heavy saline suspension of washed 72 hr. cells. The mixture was stirred slowly at room temperature for 10 to 15 min., then centrifuged at 5,000 r.p.m. for 15 min. The supernatant was decanted, and the packed cells were taken up in saline.

Material adsorbed on cells used in treating saliva was eluted by suspending the cells in saline at pH 6.5 at twice the volume of saliva originally adsorbed. The eluting mixture was stirred occasionally at room temperature for 2 hr., then the cells removed by centrifugation. The eluate was concentrated to half volume by pervaporation.

Preparation of globulin fractions of saliva was done by the following method. Whole stimulated saliva was frozen as collected and may be kept thus for months. Volumes of frozen saliva were allowed to thaw at 7° C., then filtered through glass fibers. The filtrate was passed through 2 pieces of Whatman No. 1 filter paper in a Buchner funnel, using suction. This filtrate was

placed in cellophane dialysis bags and pervaporated to one tenth of original volume by placing the bags in front of a fan. The reduced volume was centrifuged at 8,000 r.p.m. for 20 min. to pack the slight precipitate then evident. The supernatant was then placed in a dialysis bag and dialyzed for 6 hr. against a solution of $(NH_4)_2SO_4$ at pH 6.5. The $(NH_4)_2SO_4$ solution was sufficiently concentrated to produce 50 per cent saturation in the bag at equilibrium. The protein precipitate in the bag was centrifuged at 6,000 r.p.m. for 20 min., and the supernatant was discarded. The precipitate was taken up in distilled H_2O, and dialyzed 12 hr. against distilled H_2O, and then 12 hr. against 0.9 per cent NaCl at pH 7.0. The protein solution was then frozen until used. All filtrations, precipitations, and centrifugations were done at 7° C. Re-precipitation of the protein solution with subsequent washings slightly increased the bacteriolytic activity. The protein solution was commonly diluted with saline to approximately one half the volume of the saliva from which it was derived to prepare it for use in most tests.

Electrophoretic analyses of saliva and salivary fractions were performed with a Spinco Model R paper electrophoresis apparatus. The paper strips were saturated with Spinco B_2 barbiturate buffer at pH 8.6. The saliva samples were adjusted to pH 7.2, and the amount applied to the strips was 0.01 or 0.02 ml. Five milliamperes of current was applied to the electrophoresis cell for 16 hr. The strips were stained with bromphenol blue containing zinc sulfate, and fixed with sodium acetate. The strips were scanned with a Spinco Analytrol densitometer.

Sedimentation analyses were done at 59,780 r.p.m. for 1 hr. at ambient temperature. The solvent was water, and photographs were taken at 10-minute intervals.

RESULTS

It has been shown previously that growth of lactobacilli can be inhibited in a characteristic manner by the presence of saliva of caries-immune human beings (Green and Dodd[9]). It has also been shown that caries-immune saliva could inhibit acid production by lactobacilli in glucose media and could stimulate the appearance of nonaciduric cells, which were incapable of growth at pH 5. When lactobacilli were inoculated into media (see Methods) containing caries-immune (C.I.) saliva and caries-susceptible (C.S.) saliva, differences in turbidity changes were seen. The data concerning one typical lactobacillus culture are reproduced in Fig. 1. The increase in light transmission seen in curve *E* may be interpreted as a decrease in number of light-scattering particles in the medium. This interpretation has been strengthened by data from experiments where samples were removed at various intervals from tubes under observation, and the samples were plated at 1:1,000 dilution on tomato agar. Data from one such experiment are shown in Table I, where a decrease in number of viable cells occurred 2 to 6 hr. after inoculation in media containing C.I. saliva, with a concurrent increase in light transmission. This has been verified in several experiments. Lysis of some lactobacillus cells occurred in the media containing C.I. saliva.

Pooled saliva samples were gathered from at least 6 persons. During the course of this work, over 50 pooled lots each of C.I. and C.S. saliva have been separately collected and examined for biologic activity. Every lot of pooled C.I. saliva has shown the aforementioned lactobacillus-lytic property. (Also, during this time, over 400 separate cultures of lactobacilli have been tested for response to C.I. and C.S. saliva.) Of these cultures, about 80 per cent have been partially lysed on first trial with C.I. saliva, and about one third of the refractory 20 per cent were partially lysed in later trials. Of the 400 cultures, only 2 per cent were partially lysed on first trial with C.S. saliva, and only about 2 per cent of the remainder reacted in later trials.

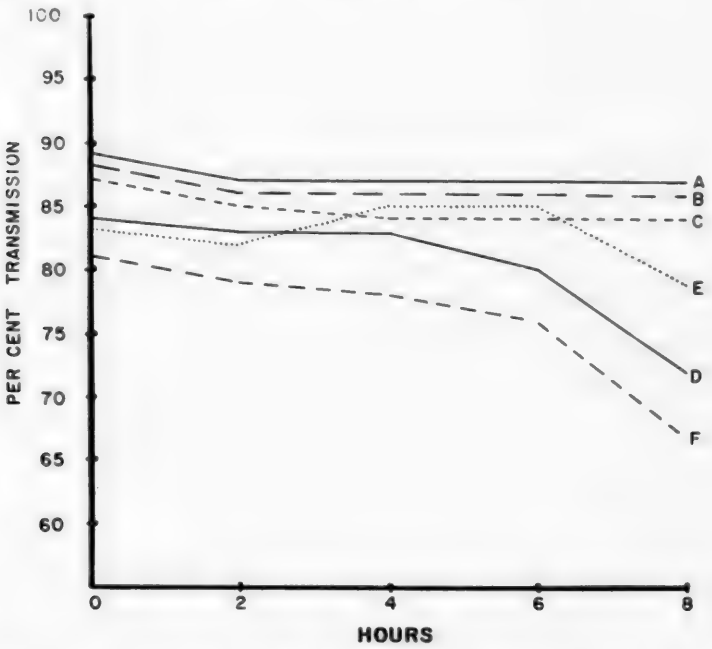

Fig. 1.—Turbidity changes in a typical lactobacillus culture in media containing saliva. *A*—uninoculated control, medium plus C.S. saliva; *B*—uninoculated control, medium plus C.I. saliva; *C*—uninoculated control, medium plus saline; *D*—inoculated tube, medium plus C.S. saliva; *E*—inoculated tube, medium plus C.I. saliva; and *F*—inoculated tube, medium plus saline.

TABLE I
NUMERICAL CHANGES IN LACTOBACILLUS CULTURES IN MEDIA CONTAINING SALIVA

CULTURE NO.	NUMBER OF COLONIES PLATED AT DIFFERENT TIMES FROM MEDIA CONTAINING:							
	CARIES-IMMUNE SALIVA				CARIES-SUSCEPTIBLE SALIVA			
	0 HR.	2 HR.	6 HR.	10 HR.	0 HR.	2 HR.	6 HR.	10 HR.
A	2,000	238*	516	3,000	1,500	2,500	3,000	7,500
B	502	800	211*	3,000	219	600	3,400	3,500
D	1,500	700*	4,000	4,000	2,500	3,000	12,000	21,000
E	5,000	10,000	9,000	10,000	12,000	16,000	16,000	14,000
F	1,200	735	360*	7,500	4,000	4,000	6,000	6,000
U	6,000	1,500*	2,500	6,000	3,000	3,000	5,000	7,500
W	156	54*	49	52	167	160	186	1,000

*Time at which lysis may have occurred.

It is clear, therefore, that this lytic effect is not an all-or-none phenomenon; it may be seen on occasion in C.S. saliva. Regarding the response of the lactobacillus cultures to the lytic effect, no correlation has been found with source (from immune or susceptible saliva), colony type, biochemical activities, or homo- or heterofermentative property.

The lytic effect on a single lactobacillus culture is reproducible, both in replicate systems and in serial passages. It has been found that cultures allowed to age for several months usually become resistant to the lytic effect of C.I. saliva. Repeated transfers may alter this (shown later in this paper).

In nearly all this work, at least 10 cultures of lactobacilli were used in each experiment to allow for culture variation. Every experiment was repeated at least once for verification. A lytically active preparation was so judged if it affected at least 40 per cent of the cultures tested; if less than 40 per cent, the experiment was inconclusive and was repeated twice more. An inactive preparation was so judged only if it failed to lyse all cultures in 2 separate trials.

Oral streptococci, isolated from both C.I. and C.S. salivas, are also liable to this sort of inhibition of both growth and acid production. This is not surprising in view of the close relationship of streptococci and lactobacilli. In this work, however, emphasis has been maintained on the effects of saliva upon lactobacilli, since these organisms occur in significantly different numbers in C.I. and C.S. salivas, and streptococci apparently do not.

Studies have been made on the environmental factors which could affect lysis of lactobacillus cultures by C.I. saliva. In these, and in all experiments reported here, comparisons were made between C.I. and C.S. salivas, and against controls containing no saliva. Media adjusted to give a resultant pH of 5.0 upon addition of C.I. saliva were inhibitory to more cultures of lactobacilli than the same system adjusted to pH 7.0. C.S. saliva in such systems was noninhibitory at either pH. This effect of pH has been examined in 3 separate experiments, using a total of 30 cultures. C.I. saliva in media at pH 5.0 inhibited all 30 cultures; at pH 7.0, only 25 were inhibited. This effect of pH could be important in consideration of the acidogenic theory of caries initiation, in which local in vivo hydrogen ion concentrations of 5.0 or less are thought to be damaging to dental enamel. The fact that the bactericidal agent in C.I. saliva is effective at pH 5.0 lends importance to its possible role in caries immunity.

A number of attempts were made to develop a quantitative test for bacteriolytic activity of saliva preparations by varying the components of the system, namely, lactobacilli cells, the nutritional medium and the saliva. Prior to testing in saliva, lactobacilli were grown in tomato juice broth, Rogosa's LBS medium (liquid modification), brain-heart infusion broth, or were harvested in saline from tomato agar. The combination of culture age and substrate at time of inoculation into saliva-containing media which gave the most consistent results was found with 72-hour cultures in tomato juice broth. Centrifugation and washing of the cells with saline improved the consistency

of their behavior. The size of the standard inoculum (0.3 ml. of a saline suspension at 0.4 optical density) is regarded as nearly a minimum for demonstration of the effect shown in Fig. 1. Quadrupling this amount of inoculum yielded nearly equivalent results. A sufficient density of cells is required to observe a decrease in density within the operational limits of the turbidimeter while a very dense cell suspension masked the bacteriolytic effect.

After trial of a number of media, it was found that brain-heart infusion broth provided the most useful nutritional base in the saliva-medium system. In most of this work, the saliva examined was used in its original concentration. The effect of concentration was studied by pervaporating saliva to various multiples of the original concentration. Twenty lactobacillus cultures were used to check the lytic property of the different concentrates. At the original concentration, 15 cultures showed evidence of lysis; at twice the original concentration, 17 were partially lysed. At 4 times, and at 10 times the original concentration, 9 and 2 cultures were lysed, respectively. The presence of nutrients (brain-heart infusion) in the system in which bacteriolytic activity occurs is not essential in all instances. A number of cultures of lactobacilli were partially lysed when placed in undiluted sterile C.I. saliva. Here again, C.S. saliva was not lytic. However, it was found that addition of the nutrient base was associated with more consistent results.

The effect of frequent transfer on susceptibility of lactobacilli to the lytic factor was studied. The 10 cultures used in this experiment were deliberately selected from old staled cultures which had been kept in a refrigerator for several months. Transfers were controlled by carrying the cultures in media in which saliva was replaced by saline. The data in Table II show that more cultures were susceptible to lysis in the later transfers in the medium plus saline than in the original transfer. The data also show what appears to be a decrease in resistance to lysis (by C.I. saliva) in those cultures carried in C.S. saliva. Conversely, at the third transfer of cultures carried in C.I. saliva, fewer cultures were lysed than at the second transfer. Possibly a selection of lysis-resistant cells occurred in this case; however, this could not be confirmed by isolation of lysis-resistant lactobacilli from the oral flora of caries-immune human beings.

TABLE II

EFFECT OF TRANSFER IN VARIOUS MEDIA UPON RESISTANCE OF LACTOBACILLUS CULTURES TO BACTERIOLYTIC EFFECT OF SALIVA OF CARIES-IMMUNE AND CARIES-SUSCEPTIBLE HUMAN BEINGS (10 CULTURES TESTED)

CULTURES TRANSFERRED IN MEDIA* WITH ADDED:	NO. INHIBITED BY C.I.† AND C.S.‡ SALIVA AT VARIOUS TRANSFERS					
	1ST TRANSFER		2ND TRANSFER		3RD TRANSFER	
	C.I.	C.S.	C.I.	C.S.	C.I.	C.S.
Saline	1	0	5	2	5	0
C.I. saliva	1	not done	6	1	3	0
C.S. saliva	not done	0	3	0	10	0

*See Methods.
†Caries-immune.
‡Caries-susceptible.

The lactobacillus-inhibitory effect of C.I. saliva is noticeable at the end of the initial stationary phase of growth (see Fig. 1). At this time some of the cells in the culture are ready to begin the first division in the new medium. Since it is obvious that some material in the saliva (the bacteriolytic factor) probably comes in direct contact with the cells, it is of interest to know whether this contact is made immediately upon placing the cells in the saliva (adsorption), or at some later time. This question was examined by inoculating several cultures of lactobacilli into tubes of the usual saliva-medium substrate and removing the cells from them by centrifugation at varying times. The cells were then re-suspended in tubes of brain-heart infusion (BHI) plus saline or saliva. It was found that lactobacillus cells adsorbed the bacteriolytic factor from C.I. saliva within 10 minutes after exposure and lysis occurred about 4 hr. after inoculation into fresh media. The data in Table III show that such treated cultures, removed from C.I. saliva, were observed to undergo the lytic phenomenon when placed in BHI plus saline, and even in BHI plus C.S. saliva. Cells similarly treated in C.S. saliva were also inhibited when placed in C.I. saliva. These results indicate that possibly only one component in C.I. saliva is responsible for this bacteriolytic property, and that nothing exists in C.S. saliva to prevent either its adsorption or its action.

TABLE III
ADSORPTION OF A LYTIC FACTOR FROM SALIVA ON LACTOBACILLUS CELLS

	LYSIS OF CELLS AFTER CENTRIFUGATION AND TRANSFER TO:		
CELLS TREATED WITH	MEDIUM PLUS SALINE	MEDIUM PLUS C.I. SALIVA	MEDIUM PLUS C.S. SALIVA
Saline	−	+	−
C.I. saliva	+	+	+
C.S. saliva	−	+	−

Adsorption of C.I. saliva (see Methods) for 10 minutes removed all detectable amounts of the bacteriolytic factor specific for untreated cells of the same culture. Streptococci, which could be inhibited by C.I. saliva in the same manner as lactobacilli, could also adsorb a homologous portion of the bacteriolytic factor. Cross-adsorption studies, using cultures of both lactobacilli and streptococci, gave the results shown in Table IV. These show that the bacteriolytic factor has multiple specificities. These observations resemble the findings obtained with some bactericidal substances in serum.

The lytic factor in C.I. saliva, adsorbed on lactobacillus cells, could be eluted from the cells (see Methods). Bacteriolytic activity was demonstrable in eluates when C.I. saliva had been treated, but not when C.S. saliva was similarly treated. The eluates exhibited lytic activity toward the cultures used for adsorption and a few others. The extracted saliva exhibited more opposite specificities, directed primarily against cultures not inhibited by the eluates.

In testing the inhibitory activity of saliva by more common methods, such as porcelain cups placed on, or wells cut in seeded agar plates, it was found that C.I. saliva produced larger inhibition zones than did C.S. saliva. This is

TABLE IV
SPECIFICITY OF INHIBITORY FACTOR IN C.I. SALIVA FOR CULTURES OF LACTOBACILLI AND STREPTOCOCCI

	INHIBITION BY ADSORBED SALIVA							
	STREPTOCOCCUS CULTURES				LACTOBACILLUS CULTURES			
ADSORBED WITH:	1	2	5	11	17	25	37	42
—	+	+	+	+	+	+	+	+
Streptococcus 1	−	−	+	−	−	−	−	−
Streptococcus 2	+	−	−	−	−	−	−	−
Streptococcus 5	−	−	−	−	+	+	−	−
Streptococcus 11	−	−	−	−	+	−	+	−
Lactobacillus 17	−	−	+	+	−	−	−	−
Lactobacillus 25	−	−	−	−	+	−	−	−
Lactobacillus 37	−	−	+	−	+	+	−	+
Lactobacillus 42	−	+	−	+	−	−	−	−

in line with the findings of others.[1, 5] It was felt that this method was more cumbersome than the turbidimeter technic, and might not be measuring the same type of inhibition, and therefore was not extensively used.

The bacteriolytic principle in C.I. saliva passes through a Selas unglazed porcelain filter. A smaller amount passes a Seitz asbestos filter pad, but all detectable activity could be removed by three passages of saliva through a Seitz filter. The bacteriolytic activity of C.I. saliva was lost with heating to 56° C. for 30 min. The bacteriolytic factor is therefore definitely heat labile, or it requires the presence of another substance which is heat labile, similar to the complement system in blood serum. Addition of unheated saliva to heat-inactivated saliva in amounts as great as 1:6 did not restore activity to the system. It is clear from this that heating does not simply destroy a component which is necessary only in small quantity for lytic activity of the saliva-medium system.

Preliminary studies of properties of the bacteriolytic factor in C.I. saliva gave the results shown in Table V. Examination of these led to the conclusion that the lytic factor is, or is closely associated with, a heat-labile protein. Accordingly, fractionation of saliva was carried out in high concentrations of salts, using the methods developed by Howe[13] and by Cohn and his associates.[14] The results of these fractionations, shown in Table VI, indicate that the lytic activity was associated with the globulin fraction of the C.I. saliva. Similar fractions obtained from C.S. saliva displayed no bacteriolytic activity. For reasons of simple manipulation, the material precipitated out by producing 50 per cent of saturation with $(NH_4)_2SO_4$ in saliva has been used as the active fraction.

TABLE V
PROPERTIES OF A LACTOBACILLUS-INHIBITING FACTOR IN C.I. SALIVA

1. Filterable—Selas porcelain bacteriologic filter
2. Adsorbed—3 passages through Seitz asbestos filter
3. Nondialyzable
4. Precipitated—by high salt concentration, ethyl alcohol, or acetone
5. Stable—freezing and thawing, lyophilization
6. Active—at pH 5.0 and pH 7.0
7. Heat labile—56° C. for 30 min.; not reactivated by subsequent addition of small amounts of unheated saliva

TABLE VI
BACTERIOLYTIC ACTIVITY OF FRACTIONS OF C.I. SALIVA

SULFATES USED*	LYSIS OF CULTURES OF LACTOBACILLI
13.5% Na_2SO_4*	+
17.4% Na_2SO_4*	−
21.5% Na_2SO_4*	−
34% of saturation of $(NH_4)_2SO_4$	+
40% of saturation of $(NH_4)_2SO_4$	+
50% of saturation of $(NH_4)_2SO_4$	+
Saturated with $(NH_4)_2SO_4$	−

*In every case, in salting out the active fractions the precipitate resulting from lower salt concentration was removed before raising concentration to next higher level.

Some effort has been spent in improving the procedure of obtaining the active protein fraction of saliva with minimum manipulation. Saliva is indeed a complex fluid, and it presents problems not encountered in fractionation of blood serum. Large volumes of mucin and perhaps mucopolysaccharides precipitate spontaneously from whole saliva upon standing, even at 7° C. Some mucin remains in solution. Stickney[15] has said that the usual technic of precipitating mucin from saliva by adding acid also removes a portion of the salivary protein. The procedure described in Methods has been used for most of this work.

The globulin fraction of C.I. saliva was found to have the same capacity as whole saliva for inhibiting growth and acid production of lactobacilli, and for eliciting nonaciduric variants under proper conditions. The bacteriolytic factor in the globulin is active at both pH 5.0 and pH 7.0, and is inactivated in 30 min. at 56° C. Saliva from which the globulins were removed did not show any evidence of the lytic factor.

Studies were made of the activity of globulin preparations at different concentrations relative to the original volume of saliva from which the protein was derived. The concentrations tested were ½, 1, 2, 3, and 5 times that found in whole saliva, assuming complete precipitation of the globulin (which of course probably did not occur). It was found that the lower concentrations (½, 1, 2) gave results approximately equivalent to whole saliva, while the inhibitory activity was markedly diminished at the higher concentrations. This resembles the observations of Maaloe,[16] who stated that an excess of normal antibody interfered with its bactericidal effects.

The possibility exists that the bacteriolytic factor under investigation is commonly possessed by both C.I. and C.S. saliva, but is not evidenced in the latter perhaps because of some accompanying "blocking" substance. To examine this, experiments were done in which fractions of both salivas were prepared and recombined with the heterologues. The results in Table VII show no evidence for this possibility, for whole C.I. saliva and C.I. globulin were active in the presence of equivalent amounts of C.S. saliva or its fractions.

An interesting observation has been made in relation to the tryptophan content of saliva fractions. Green, Dodd, and Radike[17] have shown an association of tryptophan or similar substance with the saliva of caries-immune human beings. Colorimetric analyses of whole C.I. saliva and of fractions obtained

therefrom have shown that most, if not all, the tryptophan was found in the globulin fraction, little or none in the albumin fraction. Removal of mucin by acid precipitation had no effect on the color-reacting material. It is clearly possible therefore that tryptophan or some similar compound may be intimately associated with the bacteriolytic principle in C.I. saliva.

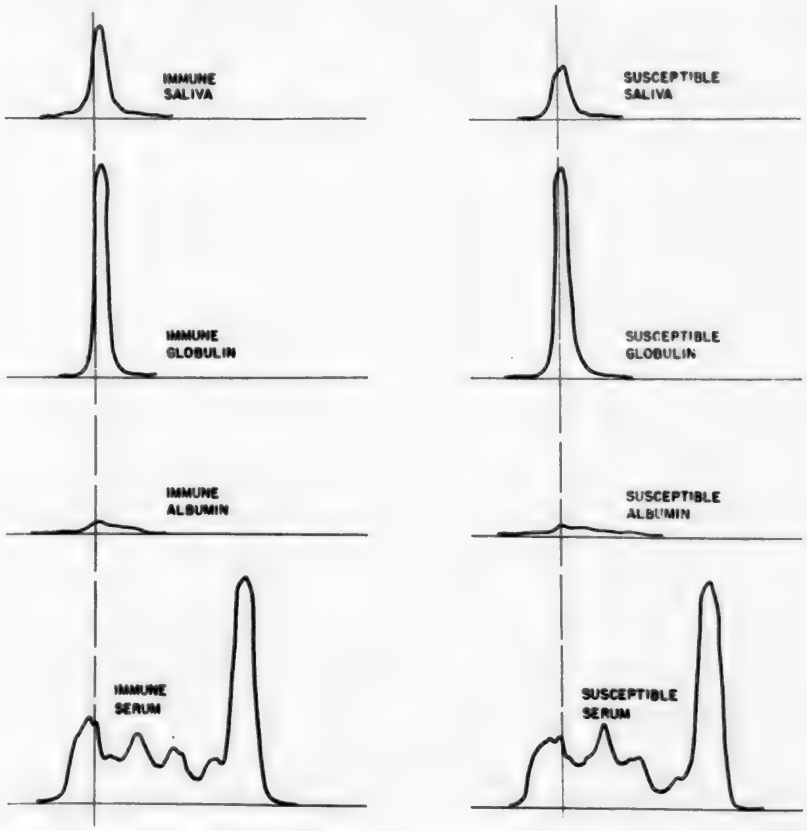

Fig. 2.—Electrophoretic studies of saliva from caries-immune and caries-susceptible individuals.

TABLE VII
BACTERIOLYTIC ACTIVITY OF FRACTIONS OF C.I. AND C.S. SALIVA, TESTED SINGLY AND IN COMBINATION

		CARIES IMMUNE		
	SALINE	SALIVA (WHOLE)	GLOBULIN	ALBUMIN
Saline		+	+	–
Caries-susceptible				
saliva (whole)	–	+	+	–
globulin	–	+	+	–
albumin	–	+	+	–

+ = bacteriolytic activity present.
– = bacteriolytic activity not present.

Paper-strip electrophoretic analyses of various saliva fractions have been made. Representative patterns from these analyses are shown in Fig. 2. The globulin fractions from both C.I. and C.S. saliva appeared to be relatively homogeneous, but the albumin fractions were not. The serum samples were included for reference purposes only, even though they were obtained from a caries-immune and a caries-susceptible person. These patterns have provided further confirmation that the active salivary fraction consists mainly of globulin.

Sedimentation analyses of saliva fractions have indicated the homogeneity of globulin preparations and the presence of at least 2 components in the albumin samples, as shown in Fig. 3. This work is preliminary, and has been done only at one pH, with one solvent (see Methods).

Preparations of salivary globulins have been used to prepare antisera in rabbits. The globulin fractions were made up to approximately the same concentration as serum globulin and were introduced intramuscularly coupled with Freund's adjuvant. Preliminary bleedings were made, and serum and antiglobulin serum were examined in precipitin tests with the homologous and heterologous antigens. Very slight specificity was seen, and then only

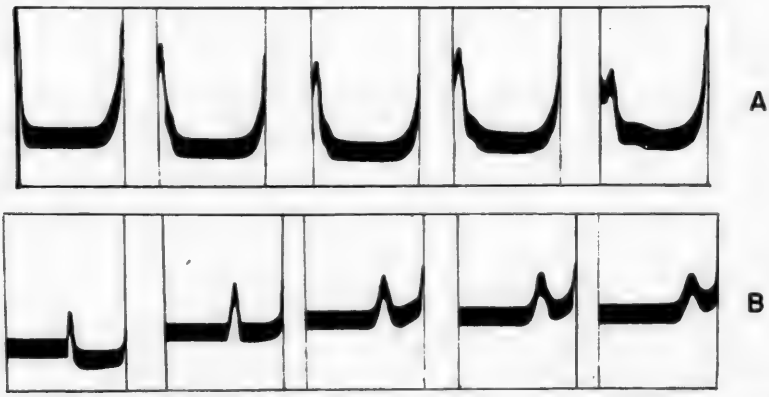

Fig. 3.—Sedimentation patterns of salivary proteins. *A*—tracing of pattern obtained with aqueous solution of total protein minus globulin; and *B*—tracing of pattern obtained with aqueous solution of gamma globulin. (Photographs taken at 10-minute intervals; displacement of peak in first photograph of *B* was due to a cell leak.)

with undiluted or 1:10 sera. Washed suspensions of 72 hr. lactobacillus cells were used to adsorb globulin preparations of C.I. and C.S. salivas. After 10-minute adsorption, the cells were centrifuged down, the supernatant removed, and the cells taken up in saline. These treated cells were examined for agglutination by the antisera in a slide agglutination test. Specificity and degree of reaction with homologous systems were improved, but were not considered completely satisfactory. It is likely that the presence of a number of serologically different globulins in the usual preparation of salivary globulin is accountable for the unsatisfactory specificity of antisera. However, the method of agglutinating treated lactobacillus cells appears promising. It has been shown that the bacteriolytic principle of C.I. saliva could be eluted from

treated cells and study of the eluted material showed that it did indeed contain globulin, precipitable at 50 per cent saturation with $(NH)_2SO_4$. Immunization of rabbits with this eluted material might engender antisera of suitable specificity for detection of the bacteriolytic factor in whole saliva by precipitin tests.

DISCUSSION

It is apparent that the bacteriolytic factor in caries-immune saliva affects lactobacilli most strikingly at the end of the initial stationary phase of growth (see Fig. 1). This particular phenomenon is rare but it has been described at least once before as a property of a nitrofuran compound (Cramer and Dodd[18]). It is not yet known whether the salivary factor attacks the susceptible cells just before or during division. It is certain that some intracellular activities at this time are peculiar to the process of initiating division, and the salivary factor therefore somehow disrupts the cell processes at this time. The salivary factor can also under proper conditions, retard multiplication in a growing culture of lactobacilli. This mechanism (inhibition) is different from the lytic effect, and may also be of importance in caries resistance.

The observation that lysis occurred at approximately the time cell division commenced in a culture, and the fact that the evidence of lysis could be amplified in serially transferred cultures, lead to the conclusion that this demonstration of lysis is partially dependent on the number of cells dividing at a given time. That is, the larger the proportion of cells which initiate division at one time, the more pronounced the lytic effect will be. The subject of synchronization of cell division has been discussed by Campbell[19] in a recent review. It is quite likely that some cells adsorb the factor and are not killed, although the difficulty in increasing the lytic effect by concentration may be in part due to the same sort of phenomenon described by Maaloe[16] in cases of excess antibody. Since it is obviously quite difficult to estimate critical concentrations for presumed instances of antibacterial activities in vivo, the results of some experiments in vitro cannot be interpreted with absolute certainty. The possibility that the salivary factor observed in vitro does actually operate in the mouth is certainly good, for the in vitro effects approximate the in vivo condition in caries-immune mouths; i.e., negligible caries activity and low numbers of lactobacilli.

The results obtained by electrophoretic analyses of saliva are similar to those obtained by Stickney[15] and Kinersly.[20] A large part of salivary protein is globulin, and probably most of this protein is derived from serum protein by "leakage" through the salivary glands. It follows that protein found in saliva could exhibit many of the activities and specificities found in serum proteins. The salivary proteins could contribute considerably to the general resistance of oral tissues to bacterial infections, admitting the great differences between serum and saliva as environments. This difference in environments could also modify protein activity beyond that found with proteins in serum.

The properties of the bacteriolytic agent described in this work show conclusively that it is not lysozyme. In his original paper, Fleming[21] showed

that lysozyme of human origin was stable when heated to 60° to 70° C. for 10 min., and was adsorbed on porcelain and paper filters. This has been confirmed with salivary lysozyme by Thompson,[4] Van Kesteren, Bibby, and Berry,[5] and Simmons.[22] Additionally, lysozyme exerts its lytic effect within minutes after contact with susceptible organisms, and has little, if any, effect on lactobacilli. The properties of this factor somewhat resemble those described by Van Kesteren, Bibby, and Berry,[5] but not those found by the other workers cited. Its spectrum of activity does not resemble that of properdin, discovered by Pillemer and his co-workers.[23] It is unlikely that this factor differs markedly from other serum-salivary proteins except in its mode of action and specificity.

An association of this bacteriolytic factor and the saliva of caries-immune human beings is claimed. It is not easy to explain why this material is present in saliva, if caries-immune individuals are peculiarly capable of producing it, or if it is common to all persons and is seen in active form only in those who are caries-immune. The converse, that is, to explain why the lytic phenomenon is only rarely detected in C.S. saliva, is no easier to explain. It has been shown that lactobacillus cells can adsorb the lytic factor, which may explain the relative lack of the factor in C.S. salivas where large numbers of lactobacilli are also present. This possibility may be negated by the fact that the lytic factor can be adsorbed on streptococcus cells also, and there are large numbers of these organisms in all C.I. salivas. Possibly the lytic factor exhibits preferential specificity for lactobacilli, or, adsorption of the factor on cells in the mouth operates differently from the observations in vitro. Insufficient data permit only speculation on these points. It would be of interest to know if the lytic factor can be traced to some protein in serum; however, such a connection may be difficult to establish if protein activity is modified by other constituents of saliva.

SUMMARY

A bacteriolytic factor was found in saliva of caries-immune human beings which was absent in most caries-susceptible salivas. The factor was found to be active against lactobacilli and streptococci, and exerted its lytic effect apparently upon cells commencing the process of division. The salivary factor had a number of effects on cultures of lactobacilli, which seemed to reduce the cariogenic potential of the organisms.

The factor could be adsorbed from saliva by lactobacillus cells and thereafter eluted from the cells in active condition. The physical properties of the factor indicated that it is a protein, and electrophoretic analyses and solubility studies showed that it was associated with the globulin fraction of saliva. The specificities and properties of the factor resemble those of some antibacterial factors in serum, and differentiate this factor from most of the salivary antibacterial principles that have recently been reported.

Thanks are due to Dr. Nancy Bigley, Dept. of Bacteriology, for electrophoretic analyses, and to Dr. Walter Frajola, Dept. of Physiological Chemistry, for the sedimentation analyses.

ADDENDUM

Since the submission of this paper, a report by Kerr and Wedderburn[24] on salivary gland secretions has appeared. The authors tested parotid and submaxillary secretions and found the former especially inhibitory to several species of bacteria. They could distinguish 2 antibacterial factors; one similar to lysozyme, the other was heat labile and active against the lysozyme-resistant organisms, which included 3 species of lactobacilli. This second inhibitory factor was apparently quite similar in properties to the lytic factor discussed here.

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BLOOD FLOW AND BLOOD PRESSURE RESPONSES TO MANDIBULAR NERVE BLOCKING AGENTS

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THE purpose of this investigation is to demonstrate the effect of nerve blocking agents on blood flow, blood pressure, and peripheral resistance, following block of the mandibular nerve in dogs. There have been many conflicting reports regarding the effectiveness, safety, duration of action, and dangers of blocking agents employed in dentistry, particularly since the introduction of vasoconstrictor agents in combination with these drugs. The addition of vasoconstrictor drugs was initiated in order to delay the rate of absorption of the solution, to decrease the amount of solution necessary to prolong the anesthetic action, and to reduce hemorrhage. There are many factors involved in evaluating the drugs currently in use. One of the primary factors which should be considered is the effect of the blocking agent on the vascular bed in the affected area before any vasoconstrictor substance is added. Gilding[1] studied the effects of unilateral procaine blocks to the forearm and reported a twofold increase to blood flow to the blocked side. Roddie, Shepherd and Whelan[2] found a twofold increase in blood flow, using the venous occlusion plethysmograph, following infiltration of the ulnar, median, and radial nerves, with 2 per cent lidocaine. Barcroft, Edholm, Foster, Fox, and Macpherson[3] reported a threefold increase in blood flow following brachial plexus blocks and suggested that this phenomenon represented interruption of sympathetic vasoconstrictor pathways. Normal blood flow, pressure, and peripheral resistance have been determined previously in the mandibular artery of mongrel dogs in this laboratory.[4]

EXPERIMENTAL PROCEDURES

Blood flow and blood pressure measurements were made on the right mandibular artery of dogs by means of direct cannulation at the level of the mandibular foramen. The animals were anesthetized by intravenous infusion of sodium pentobarbital, 33 mg./Kg., and 30 minutes were allowed to elapse before the experimental procedures were begun. The blood flow was recorded in milliliters per minute, using an electromagnetic blood flow meter similar to that described by Richardson, Denison, and Green.[5] Blood pressure was recorded by the use of a P-23 AA Statham strain gauge and Brush strain analyzer and

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ink-writing oscillograph. The peripheral vascular resistance was calculated by methods described by Green,[6] and recorded as peripheral resistance units, PRU.

The mandibular artery was exposed by an inframandibular incision, and cannulation was accomplished by insertion of polyethylene tubes with bore sizes comparable with that of the artery lumen. Calibration of the blood flow instrument was performed during measurements on each animal. The cali-

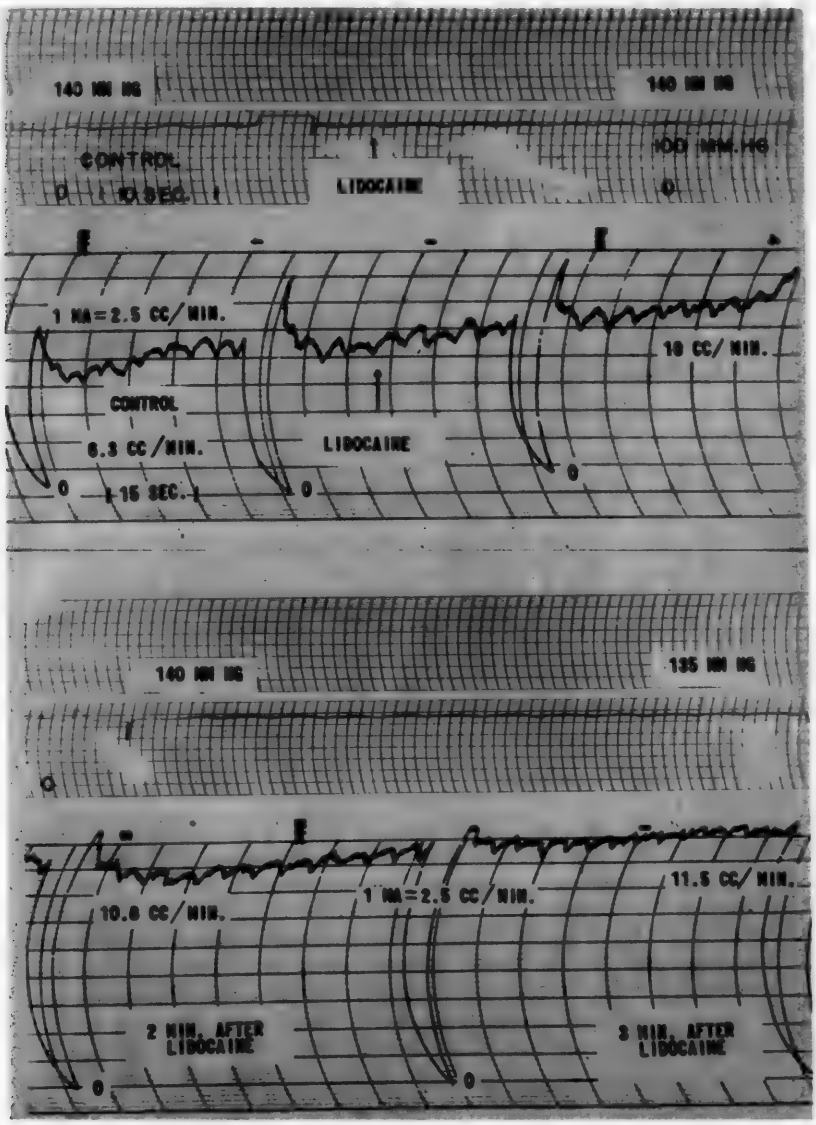

Fig. 1.—Blood pressure and blood flow responses to lidocaine placed directly on the exposed mandibular nerve reveal a steady increase in flow with pressure unchanged.

bration technic required interruption of the distal side of the cannula and collection of blood in graduated vessels for 15-second intervals with varying degrees of peripheral resistance. The calibration curve was obtained by plotting milliamperes deflection on the ordinate against determined volume flows on the abscissa.

Following determination of control levels of flow and pressure, four selected blocking agents (lidocaine, Pontocaine, procaine, and Nupercaine) were infused directly onto the exposed mandibular nerve of 20 mongrel dogs. The changes in blood flow, blood pressure, and PRU were followed for a maximum period of 10 minutes. Substitution of normal saline for the blocking agent and total section of the mandibular nerve also were performed to evaluate further the action of this nerve on the vascular bed of the mandible and to serve as additional experimental controls.

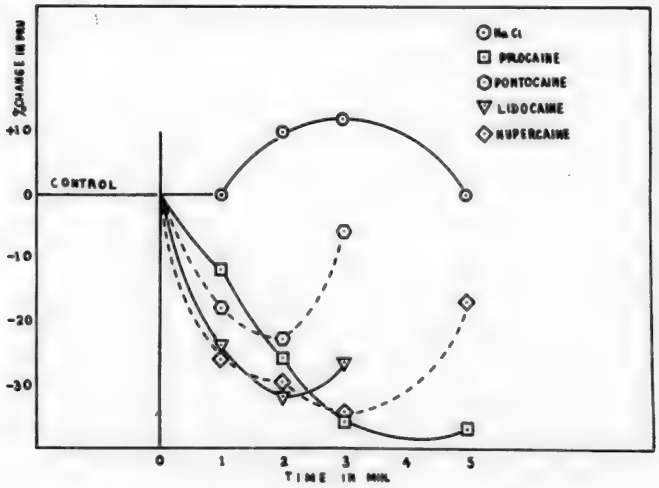

Fig. 2.—Average per cent in peripheral resistance following the application of blocking agents and isotonic sodium chloride on the exposed mandibular nerve.

RESULTS

A typical record of blood flow and blood pressure measurements is presented in Fig. 1. The steady increase in blood flow following mandibular nerve block is clearly evident, but the blood pressure is practically unchanged during the same period. Graphic analyses of the peripheral resistance changes are presented in Fig. 2. Here the general trend is toward a sharp decline in peripheral resistance followed by a somewhat slower return toward, but not reaching, the control levels by the end of 10 minutes. Table I presents the average blood flow and blood pressure and PRU values obtained from the use of each of the blocking agents, sodium chloride, and mandibular nerve section.

DISCUSSION

The decrease in peripheral resistance observed following administration of blocking agents to the mandibular nerve are primarily the results of an

TABLE I

THE EFFECT OF NERVE BLOCKING AGENTS ON BLOOD FLOW AND BLOOD PRESSURE IN THE MANDIBULAR ARTERY

AGENT	CONTROL	1 MIN.	2 MIN.	3 MIN.	5 MIN.	10 MIN.
Lidocaine						
Flow	7.1	9.3	10.3	9.8		
Pressure	128	126	125	128		
PRU	18	14	12	13		
Procaine						
Flow	6.2	6.4	7.4	8.5	9.4	
Pressure	111	101	99	98	107	
PRU	18	16	13	11	11	
Pontocaine						
Flow	4.8	5.8	6.5	6.8	5.5	6.3
Pressure	81	80	85	92	98	95
PRU	17	14	13	16	15	15
Nupercaine						
Flow	4.4	6.2	6.5	6.8	5.5	6.0
Pressure	102	106	104	105	107	105
PRU	23	17	16	15	19	19
0.9% sodium chloride						
Flow	3.0	3.0	2.8	2.7	3.0	3.0
Pressure	122	122	122	122	120	115
PRU	40	40	44	45	40	38
Nerve section						
Flow	3.6	4.8				
Pressure	93	93				
PRU	26	19				

Blood flow is recorded in cubic centimeters per minute and blood pressure in millimeters of mercury.

The data for each test agent represent the average of values obtained from 4 animals.

increase in the volume blood flow and not due to alterations in the blood pressure. The variations of the absolute values which occurred within the different experimental groups were expected, since the age, sex, breed, and nutritional status of the dogs were not controlled. For these reasons, each group of animals served as its own control, e.g., for per cent change comparisons.

The time of the course of events following blocking agent infusion was approximately the same; however, when iso-osmolar sodium chloride solution was substituted for the blocking agent, a definite decrease in blood flow occurred, associated with an increase in peripheral resistance. This occurrence may be explained by assuming that bathing the nerve tissue with the NaCl solution increased the barrage of tone impulses being conducted, resulting in an increase in the vasomotor tone of the arterioles involved.

The initial change in the peripheral resistance was followed by a period of equilibration in which the resistance tended to stabilize at a level about midway between the maximum deviation following block administration and the control level. The maximum effect always occurred within 5 minutes following block, and the flow was stabilized at the intermediate point at the end of the 10-minute experimental period. The mechanism responsible for this apparent overshoot and recompensation remains entirely obscure, and its clarification must await elucidation of the various parameters involved, such

as characterization of the nerve muscle junction at the arteriole level and the determination of the specific mechanism and site of action of the blocking agent employed.

SUMMARY

1. Control blood flow, blood pressure, and peripheral resistance have been determined in the mandibular artery of mongrel dogs.

2. Blocking agents procaine, lidocaine, Pontocaine, and Nupercaine applied directly to the mandibular nerve produced marked vasodilation and a decrease in peripheral resistance, but no significant changes in blood pressure.

3. Application of normal saline to the exposed nerve produced slight vasoconstriction, with a higher PRU, and a lower volume flow, but with no appreciable changes in blood pressure.

4. Section of the mandibular nerve produced vasodilation, lower PRU, and greater volume blood flow, without lowering blood pressure.

5. Experimental evidence is presented which suggests that the mandibular nerve is the pathway for tonic impulses which regulate the vasomotor tone of the circulatory bed in the mandibular area.

6. A basis for the evaluation of the use of vasoconstrictors in dental blocking agents has been obtained.

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SALIVARY OXIDATION-REDUCTION POTENTIALS

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INTRODUCTION

PREVIOUS workers who have attempted to measure salivary oxidation-reduction potentials electrometrically have noted unavoidable drifting of the observed potential.[1, 2, 3] However, Eisenbrandt has claimed that initial readings are reproducible.

This paper reports some preliminary observations on the nature of the drift and the validity of initial readings.

MATERIALS AND METHODS

Both resting and stimulated samples of human saliva were studied.

Measurements were made using a Pye Universal pH and millivolt meter.

The concentrated KCl/Calomel half cell had a sintered glass "liquid junction" and for the purpose of these observations was regarded as having a nominal value of +250 mv. with reference to the standard hydrogen electrode.

Three electrodes were used: (a) platinum "button," (b) platinum "spade," and (c) gold "spade." These were cleaned in one of three ways—(1) left soaking in distilled water, (2) dipped in hot chromic acid and well washed in distilled water, or (3) cleaned cathodically in the following manner. The electrode which was dipped in dilute sulfuric acid was connected to the negative pole of a 6 volt battery. Another platinum electrode, also dipped in the acid, was connected to the positive pole. A free evolution of small evenly spaced bubbles with no large one clinging to the surface of the electrode was indicative of a clean electrode. Treatment for 5 minutes was usually sufficient for platinum electrodes but gold required a longer period.

The correct functioning of the apparatus was checked by means of a freshly saturated solution of quinhydrone in standard buffer solution of pH 4.

Preliminary observations had shown that readings were affected by shaking the sample. Conditions were therefore standardized by placing the sample cup on a diaphragm vibrating 50 times per second. A modified radio earpiece was used for this purpose, into which was fed a low alternating voltage derived from the main supply which had the desired frequency.

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RESULTS

Drifting of potentials was observed in samples of resting and stimulated saliva. It was also noted in a sample of autoclaved saliva.

In a sample of constantly agitated saliva, drifting was at first rapid, gradually becoming slower, until a condition of relative stability was attained after an average of 60 minutes. The direction of the drift depended upon the treatment of the electrode prior to taking readings in saliva. It was toward a more positive potential after the electrode had been treated cathodically and toward a more negative potential after cleaning in chromic acid (Fig. 1).

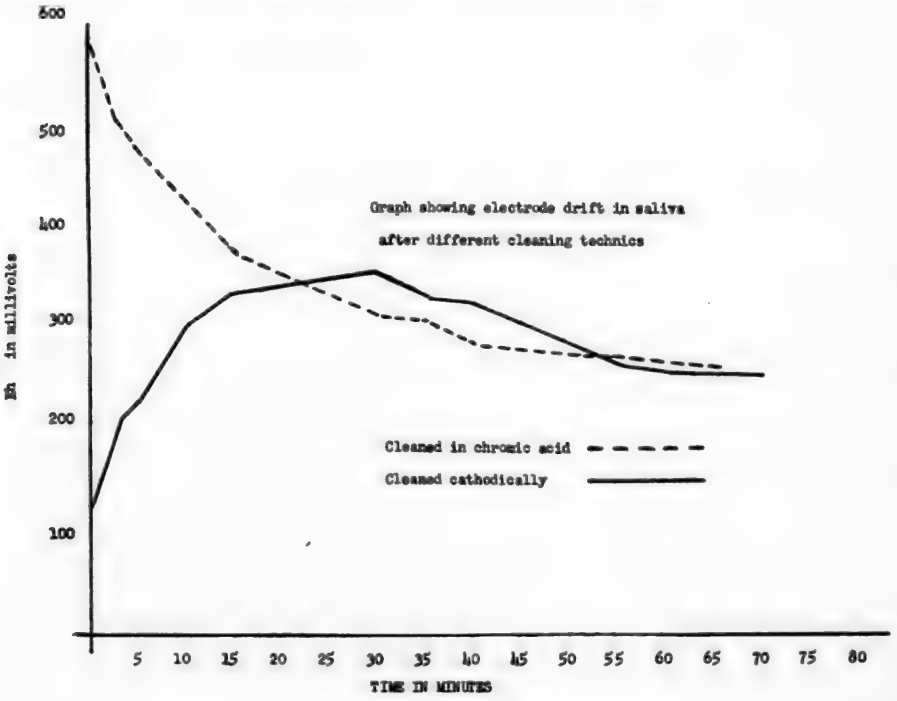

Fig. 1.

The condition of electrode stability at the end of 60 to 90 minutes could be disturbed by removing it, cleaning by whichever method had been used before, and returning it to the same sample of saliva. An "initial" reading (*B*) was then obtained similar to the first initial reading, *A* (Fig. 2). A similar period of drifting followed until a second period of stability was attained.

The most consistent results were obtained after electrodes had been soaked for 12 hours in distilled water prior to making measurements in saliva.

Different electrodes similarly cleaned which always gave the same potential for quinhydrone gave different initial readings when simultaneously immersed in the same sample of saliva (Table I). They also sometimes gave different "final" potentials.

TABLE I
INITIAL POTENTIALS OF DIFFERENT ELECTRODES IMMERSED IN QUINHYDRONE AND SALIVA

TEST SAMPLE	Eh IN MILLIVOLTS		
	GOLD SPADE	PLATINUM SPADE	PLATINUM BUTTON
Quinhydrone	+467	+467	+467
Saliva	+350	+380	+400

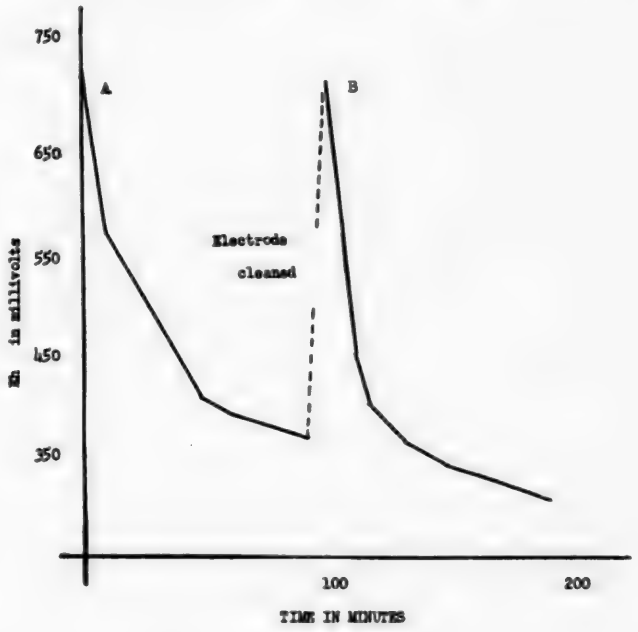

Fig. 2.

DISCUSSION

Biologic systems which do not reach immediate equilibrium with the electrode are well known. A solution of sugar slowly assumes a negative potential due to an unknown oxidation-reduction system in the solution.[4] Another example is cysteine, in which the time taken to reach equilibrium varies with the nature of the electrode, gold-plated platinum taking longer than blank platinum.[5]

Drifting is clearly a function of the electrode rather than the result of a change in the sample of saliva under observation. This is apparent from Fig. 2, although the slight shift of the curves in a negative direction may represent an actual change in the salivary oxidation-reduction potential during observation over a period twice as long as would be normal.

The variation in initial readings, which may be considerably altered by the treatment of the electrode before its immersion in saliva and to a lesser extent by the type of electrode, makes initial potentials unreliable for typing saliva.

It would also appear desirable to employ only one type of electrode during the run of any investigation.

SUMMARY

Initial readings of salivary oxidation-reduction potentials are unreliable when used to type saliva.

They are affected by the previous treatment of the electrode and to a lesser extent by the type of electrode used.

Drifting potentials are the result of some unknown function of the electrode and are not primarily due to an actual salivary oxidation-reduction potential drift during the experiment.

Relative stability is obtained after an average of 60 minutes in a sample of saliva constantly agitated.

This final reading, as opposed to the initial one, appears to be more reliable as a basis for any investigation which attempts to relate oxidation-reduction potentials of saliva to clinical conditions.

Thanks are due to W. G. Pye and Company, Ltd., for the loan of the meter and to Dr. Mattock (Chief Chemist, Electronic Instruments, Ltd.) who drew our attention to the method of cathodic cleaning.

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TRACE ELEMENTS IN ANCIENT INDIAN TEETH

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THIS study was undertaken because it was felt that teeth which had been in contact with soil for hundreds of years might have acquired many trace elements. It was hoped that knowledge of the composition of such tooth material might increase our understanding of the reactivity of dental structures.

Studies of anthropological specimens have shown that fluoride is absorbed at a slow rate. Thus, bone from the lower Pleistocene period (approximately one million years old), the middle Pleistocene, and the upper Pleistocene (approximately half a million years old) have been estimated to contain 2.0, 1.5, and 0.5 per cent fluoride.[1] Because of variations in the fluoride content of soil from different areas, different fluoride concentrations have been reported for specimens originating from the same period, but generally higher concentrations of fluoride are found in the older specimens.[1]

In addition to fluoride, other constituents of ancient teeth and bone including magnesium, aluminum, iron, carbonate, sulfate, carbon, and nitrogen have been studied by various workers.[1-6] It is believed that calcium may be partially replaced by iron, aluminum, and magnesium, and carbonate by fluoride, sulfate, and oxygen.[4] Calcium phosphate may also be exchanged with calcium carbonate; this reaction is believed to follow the law of mass action, i.e., excess of environmental carbonate will replace phosphate and vice versa.[4] Accordingly, carbonate values higher than, similar to, or less than those of contemporary bones and teeth have been reported in ancient specimens.[3-6]

Generally, fossilization results in removal of organic material and increase in calcium phosphate or calcium carbonate. The water content also tends to diminish with age.[3] From the foregoing it is evident that marked chemical changes may take place in buried teeth and bones.

In freshly extracted teeth, it has been found that the acquisition of many trace elements is confined primarily to the surface layers.[7, 8] To determine whether penetration of trace elements into the bulk of the tooth is a question of time, the concentrations of these elements were determined in surface and subsurface material of ancient teeth. The findings were correlated with comparable data available on contemporary teeth.

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MATERIALS AND METHODS

The tooth samples included 12 Pueblo Indian permanent teeth and 23 Indian Knoll permanent teeth. The 12 Pueblo Indian teeth were orginally discovered in Pueblo Bonito, New Mexico.[9] They are believed to be contemporaneous with the ruins which, on the basis of tree rings, have been dated between 919 and 1130 A.D.[10] These ruins, which are the oldest definitely dated Pueblo remains in the southwest United States, sheltered about 1,200 individuals. It is believed that the settlement was abandoned because the soil became unproductive. The ruins, including some human bones and skulls, have been covered by sand and earth for centuries. The teeth were intact and well preserved except that the roots were stained rusty brown.

The Indian Knoll teeth were found in excavations from an aboriginal settlement situated close to the bank of Green River in Kentucky. They have been estimated to be approximately 5,000 years old by radiocarbon dating.[11] The Indians were food gatherers and hunters and not agriculturists as the Pueblos. Judging from shell mounds at the site of the settlement, a substantial part of their food was shell fish caught in the river.[12] The teeth were buried in sandy soil. They showed no discoloration and were remarkably well preserved.

Five or six successive layers were ground from the enamel with a diamond stone and collected over a porcelain filter by means of suction.[7] The first and second layers were about 0.1 mm. thick, the third and fourth layers were somewhat thicker, and the fifth layer represented the bulk of the remaining enamel. During the grinding, some of the enamel chipped off. The chips were pooled and analyzed separately. Other portions of enamel, fractured in shipping and representing whole enamel, were also analyzed. In addition, 6 successive layers of the roots were ground off. The first layer represented cementum, while the other layers consisted primarily of dentin. The fluoride was determined on samples ashed at 575° C. Fluoride was separated by the distillation method of Willard and Winter,[13] and the fluoride content of the distillate determined by Smith and Gardner's titration method,[14] which is patterned after the salt-acid thorium nitrate procedure of Williams. Carbondioxide was determined on dry weight samples according to Sobel's modification of the van Slyke-Folch manometric method[15] using McFayden's reagents and factors.[16] Manganese, zinc, silicon, lead, copper, silver, tin, strontium, iron, and aluminum were measured by a spectrographic method employing the direct-current carbon arc and a Bausch and Lomb medium quartz spectrograph. Each element was determined separately, specific information being given in Table I. The sample was placed in the negative electrode and the arc operated at 130 volts line potential and a current of 15 amperes. For readily measurable concentrations, the sample of tooth material was added to the crater in the electrode as 0.1 ml. of dilute hydrochloric acid solution containing 1 mg. of material. In general, analyses were done in triplicate. For low concentrations the larger sample amount, as indicated, was placed dry in the crater and then hydrochloric acid was added. Zinc, however, was deter-

mined on the dry sample without using hydrochloric acid. The exposure was adjusted by means of wire mesh screens, the lowest transmission being required for strontium. Spectrum line intensities were evaluated by means of a rotating sector method. The highest purity spectrographic electrodes were used for most of the elements measured and, with the exception of iron, no trouble was experienced with impurities in the electrodes. Because of sampling errors and the random errors inherent in the carbon arc method of excitation, the accuracy of measurent is about ± 20 per cent.

TABLE I

ELEMENT	Pb	Sn	Cu	Zn	Si	Mn	Ag	Sr	Fe	Al
Spectrum line (angstroms)	2,833	2,840	3,274•	3,345	2,882	2,576	3,281	4,078	2,599	3,093
Amount of sample (milligrams)	1-10	1-10	1-3	1-5	1-3	1-3	1-3	2-5	1-5	1-3
Internal standard	Bi	Pt	Pt	Pt	Pt	Pt	Cu	Ag	Pt	Pt
Internal standard spectrum line	2,898	2,651	2,651	2,651	2,651	2,651	3,248	3,281	2,651	2,651
Amount of internal standard (micrograms)	0.25	40	100	40	10	10	0.1	0.5	20	40
Arc burning time (minutes)	1.5	3	3	2	3	2	2	3	3	2.5
Minimum detectable concentration (ppm)	20	0.5	5	60	2	1	0.2	10	10	5

RESULTS

The results obtained on the enamel of the Pueblo Indian teeth are given in Table II. All the trace elements included in the analysis occur in greater concentrations in the outer rather than the inner enamel. The enamel chips which represent either whole enamel or enamel from which the outer surface had been ground show concentrations of trace elements similar to those occurring in the fourth or fifth enamel layer.

In the external enamel, fluoride occurs in the greatest concentrations, followed by zinc, silicon, manganese, lead, silver, copper, and tin. Zinc is the

TABLE II
CONCENTRATIONS OF F, Zn, Si, Mn, Pb, Ag, Cu, Sn, AND CO_2 IN SUCCESSIVE LAYERS AND IN CHIPS OF ENAMEL FROM PUEBLO INDIAN TEETH*

LAYERS	F	Zn	Si	Mn	Pb	Ag	Cu	Sn	CO_2
No. 1	1,640	1,300	870	90	80	25	5	1.0	1.75
No. 2	675	1,300	48	35	25	20	2	0.0	1.81
No. 3	408	1,300	25	20	23	15	3	0.0	2.01
No. 4	232	800	32	35	13	15	2	0.0	2.07
No. 5	180	740	32	42	14	13	1	0.0	2.44
Chips whole enamel	228	700	33	27	12	5	1	0.0	2.52

*F in ppm of ash, the remaining elements in ppm dry weight and CO_2 in percentage dry weight.

only trace element which occurs in high concentrations throughout the enamel. Of the remaining elements, fluoride, silicon, manganese, and lead occur in appreciably greater concentrations in the outer than the inner enamel. Silver, copper, and tin are also higher in the surface layers but the magnitudes are small. Carbonate has the smallest concentration in the outer enamel and increases toward the dentin.

Table III shows the concentration of trace elements in 6 successive layers from the roots of the Pueblo Indian teeth. With the exception of manganese, which has an even distribution, the various trace elements again occur in greater concentrations in the surface than in the subsurface material. Fluoride shows a consistent decrease in concentration from the first to the sixth layer while silver and silicon tend to reach a plateau beginning at the second or third layer. Lead and zinc show the smallest concentration in the fourth layer and a gradual increase in the fifth and sixth layer. Generally, the concentrations of trace elements are greater in dentin than in enamel. This is particularly true of silicon, manganese, fluoride, and zinc. As was the case with enamel, tin is only detectable in the outermost layer.

TABLE III
CONCENTRATIONS OF F, Zn, Si, Mn, Pb, Ag, Cu, AND Sn IN SUCCESSIVE LAYERS FROM ROOTS OF PUEBLO INDIAN TEETH*

LAYERS	F	Zn	Si	Mn	Pb	Ag	Cu	Sn
No. 1	4,020	5,300	2,500	400	80	50	25	1.0
No. 2	3,210	2,100	1,360	400	25	10	5	0.0
No. 3	2,660	1,500	330	610	20	10	5	0.0
No. 4	1,940	1,100	340	400	13	12	4	0.0
No. 5	1,330	1,500	330	450	38	15	1	0.0
No. 6	934	1,700	320	500	44	15	1	0.0

*F in ppm of ash, remainder in ppm of dry weight.

Tables IV and V show the findings in the first, third, and sixth layers of enamel and dentin from the Indian Knoll teeth. Although these teeth are about 6 times older than the Pueblo Indian teeth, the distribution of trace elements from the surface inward is similar in both groups, with higher concentrations in surface than subsurface enamel and dentin. The following differences may be observed between the 2 groups: (1) the enamel surface accumulation of fluoride, silicon, and manganese is more marked in the Indian Knoll than in the Pueblo Indian teeth, (2) the Pueblo Indian teeth contain greater amounts of zinc, silicon, manganese, and silver, and smaller amounts of copper than the Indian Knoll teeth, and (3) the dentin in the Pueblo Indian teeth has

TABLE IV
CONCENTRATIONS OF F, Zn, Si, Mn, Pb, Ag, Sr, Cu, Sn, Fe, AND Al IN FIRST, THIRD, AND SIXTH LAYER AND IN CHIPS OF ENAMEL FROM INDIAN KNOLL TEETH (5,000 YEARS OLD)*

LAYERS	F	Zn	Si	Mn	Pb	Ag	Sr	Cu	Sn	Fe	Al
No. 1	2,030	830	1,100	240	85	3.0	210	27	3.3	400	380
No. 3	542	500	30	11			200	25		50	60
No. 6	275	400		24	50	0.6	300	14	2.7	50	46
Chips	160	100	15	11	60	0.7	200	40	3.3	40	35

*F in ppm of ash, remainder in ppm dry weight.

much greater concentrations of fluoride, zinc, silicon, and manganese than the enamel. In the Indian Knoll teeth, the difference in composition between enamel and dentin is much less marked. Strontium, iron, and aluminum were measured only in the Indian Knoll teeth.

TABLE V
CONCENTRATIONS OF F, Zn, Si, Mn, Pb, Ag, Sr, Cu, Sn, Fe, AND Al IN FIRST, THIRD, AND SIXTH LAYER AND IN CHIPS FROM ROOTS OF INDIAN KNOLL TEETH*

LAYERS	F	Zn	Si	Mn	Pb	Ag	Sr	Cu	Sn	Fe	Al
No. 1	2,580	460	250	64	135	0.6	360	73	3.3	1,200	320
No. 3	1,312	340	50	35			290	25		100	90
No. 6	536	100	35	20	67	0.6	310	35	3.3	60	66
Chips	940	570	37	31	60	1.0	330	53	3.3	80	80

*F in ppm of ash, remainder in ppm dry weight.

DISCUSSION

Contrary to expectation, the concentrations of trace elements were conspicuously high only in the outer portions of enamel and dentin, while the remainder of the teeth contained only small amounts. A similar pattern of distribution has been found in the enamel of contemporary teeth with regard to fluoride, lead, and tin. In view of the great age of the teeth, it was anticipated that the trace elements would have diffused from the surface to the subsurface material and that the distribution would be nearly the same in different portions of enamel and dentin. The failure of any significant transfer of ions from the surface inward may well be related to the tendency of the water content of calcified structures to decrease with age.[3] The exceedingly rapid exchange reactions which may take place in vivo in the inorganic portion of calcified structures are believed to be confined to the surface of the apatite crystals and to be due to the presence of a hydration shell.[17] Because of the age of the present material, it is possible that hydration was minimal and that conditions for ionic transfer thus were unfavorable.

The remarkable similarity between the distribution of fluoride in this ancient tooth material and that of contemporary teeth is demonstrated in Table VI. Here the fluoride data of the Indian teeth are compared with corresponding data obtained on contemporary teeth, aged under 20 and over 50 years, from an area with 1 ppm of fluoride in the drinking water, and over 50 years, from an area with 5 ppm in the drinking water. The data on the contemporary teeth have been taken from a paper published elsewhere.[18] The fluoride content of enamel can increase appreciably during the relatively few years of adult life. Remarkably high concentrations of fluoride may be acquired by the enamel in vivo when fluoride is present in concentrations of 5 ppm in the drinking water. The fluoride figures for the enamel of the Indian teeth are quite similar to those of the enamel in the teeth over 50 years of age from the area with 1 ppm of the fluoride in the drinking water.

Obviously these ancient Indian teeth have not been exposed to such high fluoride concentrations as 5 ppm. Fluoride concentrations of 1 ppm or more are common in the drinking water in the northwest part of New Mexico[19] where

TABLE VI
CONCENTRATIONS OF F (PPM OF ASH) IN 5 SUCCESSIVE LAYERS OF ENAMEL FROM ANCIENT INDIAN TEETH AND CONTEMPORARY TEETH FROM AREAS WITH 1 AND 5 PPM F IN DRINKING WATER (AGE OF CONTEMPORARY TEETH, UNDER 20 AND OVER 50 YEARS)

	ANCIENT TEETH		CONTEMPORARY TEETH		
			1.0 PPM F IN WATER		5.0 PPM F IN WATER
LAYERS	PUEBLO 800 YR.	KNOLL 5,000 YR.	<20 YR.	>50 YR.	>50 YR.
No. 1	1,640	2,030	889	1,552	3,370
No. 2	675	863	363	727	1,710
No. 3	408	542	255	328	1,124
No. 4	232	393	171	227	926
No. 5	180	327	133	219	811

Pueblo Bonito is located. Therefore, it is likely that the Pueblo Indians were exposed to some fluoride in vivo unless their main source of drinking water was collected rain water. No information is available concerning the concentration of fluoride in water from the location where the Knoll Indians lived, but 1 ppm has been found in well water within 40 miles of this area. Regardless of the amount of fluoride acquired in vivo by these teeth, it is evident that only small amounts of fluoride have been taken up during the several hundred years of contact with soil.

Table VII shows a comparison of the concentration of lead and copper in the enamel of the ancient Indian teeth and contemporary teeth from Rochester, New York, under 20 and over 50 years of age.[18, 19] It is apparent that the concentration of lead is appreciably lower in the ancient Indian than in contemporary enamel. The copper values of the Pueblo Indian enamel are smaller than, while those of the Knoll Indian teeth are similar to, the values found in contemporary enamel.

TABLE VII
CONCENTRATIONS IN PPM DRY WEIGHT OF LEAD AND COPPER IN THE ENAMEL OF ANCIENT INDIAN TEETH AND CONTEMPORARY TEETH FROM ROCHESTER, N. Y., UNDER 20 AND OVER 50 YEARS OF AGE

ENAMEL LAYER	LEAD PPM				COPPER PPM			
	ANCIENT		CONTEMPORARY		ANCIENT		CONTEMPORARY	
	PUEBLO	KNOLL	<20 YR.	>50 YR.	PUEBLO	KNOLL	<20 YR.	>50 YR.
1	80	85	350	550	5	27	20	20
2	25	—	220	460	2	—	15	22
3	23	—	180	420	3	25	30	13
4	13	—	74	310	2	—	25	12
5 or 6	14	50	35	156	1	14	—	—

Tin is another trace element which has been found in greater concentrations in the enamel of contemporary teeth[20] than in this ancient material. Detailed data of the distribution of other trace elements in contemporary enamel are not available in the literature but findings in this laboratory suggest that silicon, manganese, iron, and aluminum occur in greater concentrations in the outer enamel of the ancient than in contemporary teeth. The high concentrations of silicon in the outermost layers of enamel and dentin are particularly interesting in view of the observations that silification of fossil bone is rare.[4]

This reaction may generally be surface bound and thus difficult to detect when bulk analysis of specimens is employed. In subsurface enamel the concentrations of silicon, manganese, iron, and aluminum appear to be within the range found in contemporary teeth. Strontium appears to be a little greater in the roots than the enamel, to have a relatively even distribution, and to be in the range of values for contemporary teeth. Zinc concentrations in enamel of the Indian teeth are high compared to figures given in the literature but comparable concentrations have been found in this laboratory in the enamel of contemporary teeth. The concentration and distribution of carbonate (CO_2) in the Pueblo Indian enamel is similar to that of contemporary teeth.[21]

Studies on the distribution of fluoride in roots of contemporary teeth[22] also show a similar pattern to that obtained on the present material. The concentrations of zinc, silicon, and manganese in roots of the Pueblo Indian teeth are sufficiently high compared to contemporary teeth to suggest appreciable acquisition from the soil. However, the marked difference in the concentrations of these elements and of silver and copper in the Pueblo and Indian Knoll teeth may in part have developed in vivo as a result of differences in the diet of the two tribes.

All trace elements found in the ancient Indian teeth have been detected in contemporary tooth material. The elements which will appreciably combine with dental structures include fluorine, zinc, silicon, lead, aluminum, and iron. Fluoride enters the apatite crystal by exchanging with hydroxyl or carbonate, and lead may replace calcium.[23] The reactions of zinc, silicon, aluminum, and iron with calcified structures are not well understood. However, all these elements react similarly in that acquisition is confined to the outer portion of the tooth and that penetration into the tooth is an exceedingly slow process.

SUMMARY

1. The distributions of F, Mn, Zn, Si, Pb, Cu, Ag, Sn, Sr, Fe, Al, and CO_2 were studied in Pueblo Indian (800 years old) and Indian Knoll (5,000 years old) teeth.

2. All trace elements occurred in greater concentrations in the outer than in the inner portion of the enamel. Fluoride was found in greatest concentration in the external enamel, followed by Zn, Si, Mn, Pb, Ag, Cu, and Sn.

3. In the roots, as in the enamel, the various trace elements occurred in greater concentration in surface than subsurface material. The only exception was manganese which had an even distribution. Generally, the concentrations of trace elements were greater in dentin than enamel.

4. There was a remarkable similarity in the distribution of many trace elements between ancient American Indian teeth and contemporary American teeth.

5. Contrary to expectation, there was little penetration of trace elements into the bulk of the tooth structure even in the 5,000-year-old teeth.

We are indebted to Dr. George E. Kosel and Dr. Karl V. Sarnas for contributing the teeth used in this study.

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CHANGES IN MICROHARDNESS OF ENAMEL FOLLOWING TREATMENT WITH LACTATE BUFFER

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EARLIER work[1, 2, 3] in this laboratory with the artificial mouth has attempted a qualitative demonstration of the etiology of the carious process. More recently[4] a semiquantitative method was reported in which changes in microhardness were used for measuring the rate of in vitro carious attack. However, because of the variability of the rate of surface softening, the results from many teeth were averaged in order to establish trends.

The present study was undertaken to determine the rate of change of enamel hardness under simpler conditions, with the purpose of demonstrating particularly whether variation in the resistance of teeth was an important factor. The rate of softening of teeth produced by a lactate buffer at a constant temperature and constant pH provides a simpler system than the artificial mouth to test the possible variability of individual teeth. This paper presents data on the rate of softening of human teeth exposed to 0.001 M lactate buffer at pH 5.0, 35° C. for periods up to 10 hours. The effect of topical fluoride on the rate of softening is also described.

EXPERIMENTAL

Human anterior teeth for this study were collected from dentists in Birmingham, Alabama, where the fluoride content of the drinking water is 0.0 to 0.3 ppm. After extraction, the teeth were stored in distilled water and thymol to prevent drying and to inhibit bacterial growth. Prior to measuring the hardness, the enamel surface was cleaned with water and a rubber prophylaxis cup, using very light pressure.

Hardness determinations were made as described in a previous paper[5] using a Kentron microhardness tester and a 500 gram load applied for 10 sec. Ten impressions were placed on each enamel surface at the beginning of the experiment and after each treatment period.* The average for these ten

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*Because of the non-planarity of intact enamel surfaces, the hardness measurements were made by adjusting the tooth and indentor at each reading and not solely at the first reading as recommended for plane surfaces. This results in an initial impression before the regular impression is superimposed upon it. With the standard metal hardness block, this procedure results in a reduction of the hardness values of about 70 K. N. for hardnesses around 600 K. N. For a ground tooth surface, the procedure used in the present work gave an average hardness of 269 K. N., whereas the recommended procedure gave 318 K. N. The hardnesses for teeth reported previously from this laboratory are then presumably reproducible but somewhat lower than they might have been if the method used for metal surfaces had been possible.

impressions was taken as the hardness value. Groups of 4 to 6 teeth at a time were exposed to 1 L. of 0.001 M sodium lactate buffer at pH 5.0 maintained at 35° C., and stirred continuously. The acrylic boxes carrying the teeth were sealed to the bottom of the beaker by the use of a sticky wax, so that the experimental surface lay face-up. At predetermined intervals the teeth were removed from the buffer solution, washed in distilled water, and stored in an atmosphere saturated with water until their hardness had been determined. They were then exposed to a fresh buffer solution for an additional period, and the change of hardness determined.

During each experiment, samples of the buffer were removed and checked to ensure that the pH remained constant. If a deviation of more than 0.2 pH unit occurred, the buffer was considered exhausted and replaced by a new solution. This was only found necessary in treatment over the longer time intervals. This set of conditions was established after an extensive study of the effect of pH and especially lactate concentrations and seems to be optimal for the purpose. At higher concentrations of lactate, the rate of softening proceeded too rapidly, surface loss was apparent, and satisfactory measurements could not be made. Lower concentrations had insufficient buffer capacity to maintain the initial pH.

Ground surfaces were prepared by a modification of the technic described by Hammarlund-Essler[6] using increasingly fine grits of silicon carbide paper.* Final polishing was effected with emery polishing paper,† grit No. 4/0.

In testing the effect of fluoride, the teeth were placed in acrylic boxes and exposed for 2 hours to 500 ml. of a 2 per cent solution of NaF at pH 6.5 and 35° C. The fluoride-treated teeth were then exposed to a lactate buffer similarly to the control group of teeth.

RESULTS

Intact Surfaces.—Forty intact teeth were exposed to the lactate buffer during 53 experimental runs and the results are shown in Fig. 1. Time intervals of 2, 3, 4, 6, 8, and 10 hours were used. Because of the curvature of the tooth surfaces, the number of symmetrical indentations that could be made is limited and usually only two time-periods could be employed for each tooth.

It will be noted from Fig. 1, which gives the detailed data for each tooth, that the over-all pattern is a fairly constant linear rate of decrease of hardness of intact enamel for the 10-hour period. In Table I the average percentage decrease of Knoop hardness number per hour at each time interval and over the entire period are tabulated. The over-all average was 5.3 per cent per hour.

Similar studies were made of the rate of softening of the intact surface enamel of cattle incisor teeth. The results reported in Table I show that the softening rate is somewhat greater than that found for human teeth. The

*Minnesota Mining and Mfg. Co., St. Paul, Minn.
†Norton Berh-Manning, Troy, N. Y.

TABLE I
AVERAGE RATE OF SOFTENING OF ENAMEL ON EXPOSURE TO LACTATE BUFFER

	TIME IN HOURS						
	2	3	4	6	8	10	AVERAGE
			Intact Enamel (Human)				
KN drop/hr.	19.5	16.0	22.0	23.0	20.0	19.0	19.9
% KN drop/hr.	5.3	4.5	6.1	5.7	5.4	5.1	5.3
			Ground Enamel (Human)				
KN drop/hr.	22.9	20.2	22.5	22.7	23.1	22.0	22.2
% KN drop/hr.	7.3	6.4	7.9	7.8	7.5	6.9	7.3
			Intact Enamel (Bovine)				
KN drop/hr.	35.9	—	—	27.6	—	21.8	28.4
% KN drop/hr.	10.0	—	—	7.4	—	6.2	7.9

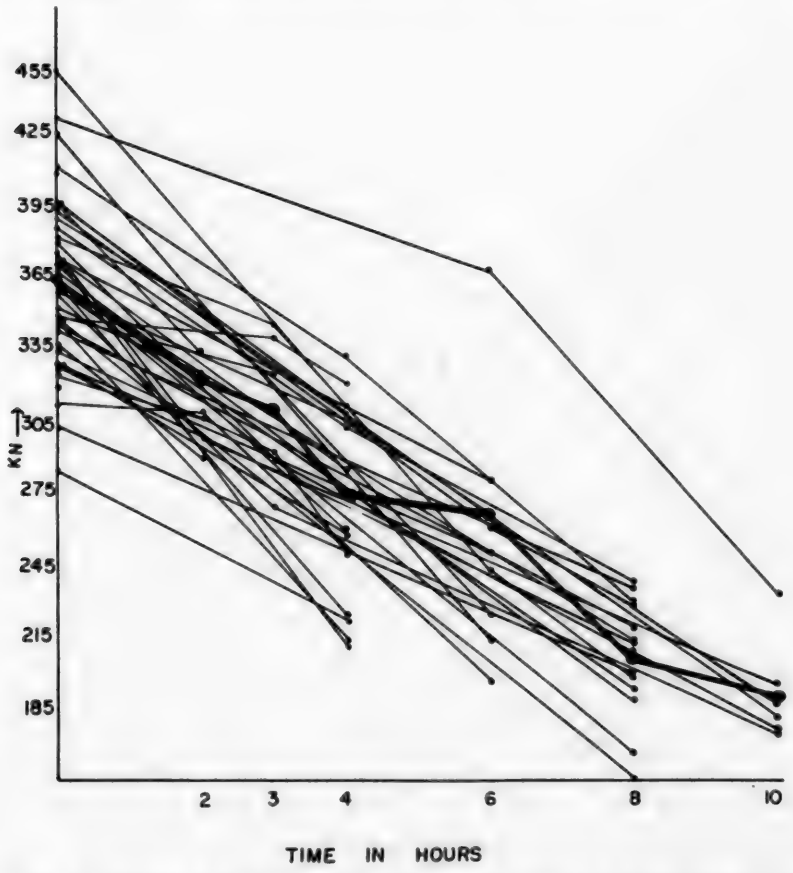

Fig. 1.—Decrease in hardness of intact enamel on exposure to lactate buffer. Temperature, 35° C.; pH, 5.0; 0.001 M. Light lines indicate individual teeth; heavy lines represent average decrease.

cattle teeth are similar to those of human beings but are much larger. Their average initial hardness of 356 ± 28 K. N. for 9 teeth was nearly the same as for human teeth (367 ± 35 K. N.) as measured in this paper.

Ground Surfaces.—Twenty-two teeth which had been used for measurements of the resistance of their intact surfaces were ground and exposed to lactate buffer during 54 experimental runs. For the ground surfaces, the spread of points (Fig. 2) was less than with the intact surfaces, and the standard deviation of the individual readings was less. The fall in hardness was again found to proceed at a constant linear rate (Table I) of 7.3 per cent per hour which was more rapid than that for the intact surfaces (5.3 per cent per hour).

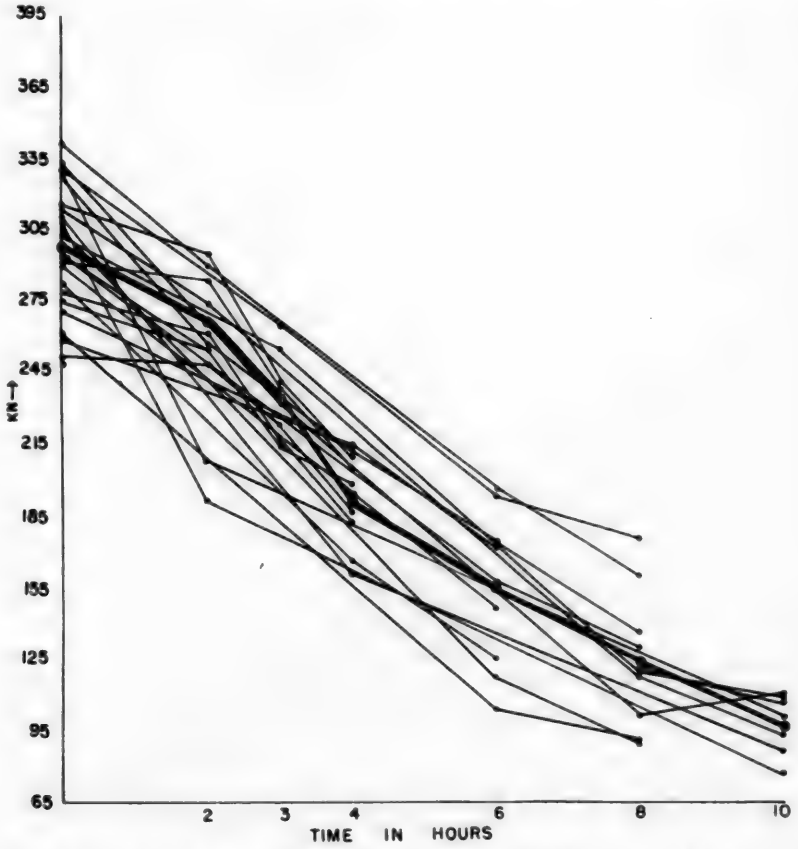

Fig. 2.—Decrease in hardness of ground enamel on exposure to lactate buffer. Temperature, 35° C.; pH, 5.0; 0.001 M. Light lines indicate individual teeth; heavy lines represent average decrease.

Effect of Fluoride on the Softening of Enamel Surfaces.—The treatment of enamel surfaces with a 2 per cent solution of sodium fluoride produced no significant change in the absolute hardness. A group of 6 teeth with intact enamel surfaces was found to have an average hardness of 397 K. N., and after the application of sodium fluoride, the average hardness was again found to be 397 K. N. Likewise, a group of 6 teeth with ground enamel surfaces

had an average initial hardness of 246 K. N., and after fluoride treatment the average hardness was 244 K. N. Any changes of the individual teeth in hardness, arising solely from fluoride treatment were within the standard deviation of the initial hardness reading, as was found previously in this laboratory.[4] These hardness changes were both greater and less than the original value and did not indicate a definite trend.

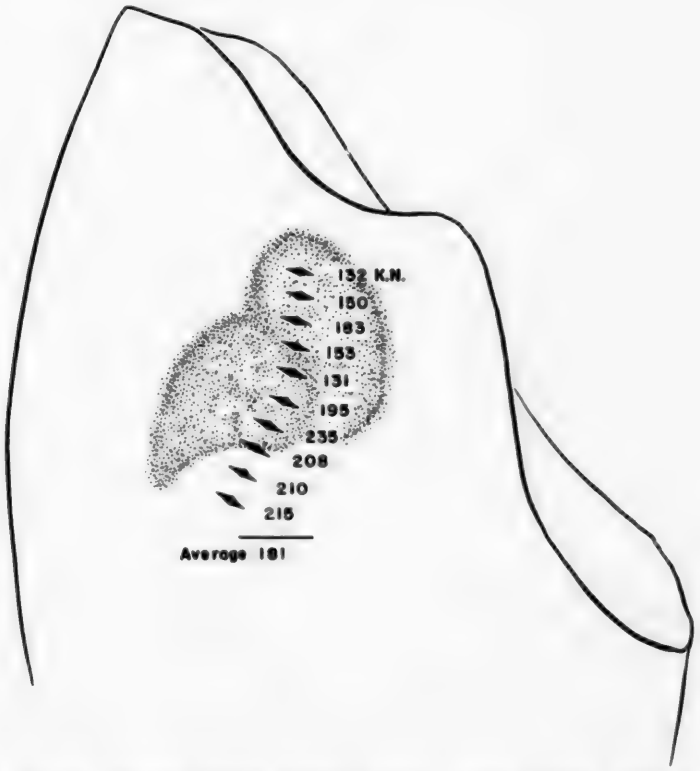

Fig. 3.—Knoop hardness number readings across a natural carious lesion on the interproximal of a canine. The dotted area had a yellowish-white color, but showed no signs of loss of surface.

Phillips and Swartz[7] found a slight increase in the hardness of ground enamel after fluoride treatment (5.1 per cent with NaF and 7.1 per cent with SnF_2). An in vivo study,[8] using young dogs, indicated an increase in enamel hardness of 13 per cent, following eight topical applications of sodium fluoride. The results of the present work do not agree with these earlier reports.[7, 8]

After exposure for 8 hours to a lactate buffer, 14 teeth with intact enamel surfaces showed a decrease in hardness of 5.4 per cent per hour as compared to 5.1 per cent per hour for a group of 9 teeth which had been fluoride treated (Table II). This difference was not significant ($P > 0.1$).

TABLE II
AVERAGE EFFECT OF FLUORIDE TREATMENT ON THE RATE OF SOFTENING OF HUMAN ENAMEL AFTER 8 HOURS

TREATMENT	INITIAL HARDNESS (KN ± S.D.)	HARDNESS AFTER LACTATE TREATMENT (KN ± S.D.)	DROP IN HARDNESS (KN)	% DROP (% INITIAL KN)	KN DROP/HR.	% KN DROP/HR. ± S.D.
			Intact Human Enamel			
None	369 ± 36	207 ± 25	162	43.5	20.0	5.4 ± 1.0
2% NaF	377 ± 36	223 ± 38	154	41.1	19.3	5.1 ± 0.8
			Ground Human Enamel			
None	310 ± 22	124 ± 23	186	59.9	23.1	7.5 ± 0.9
2% NaF	267 ± 28	141 ± 12	126	47.2	15.7	5.8 ± 0.9

For the ground teeth, the untreated surfaces showed an average decrease in hardness of 7.5 per cent per hour for a group of 10 teeth. On the other hand, the fluoride-treated ground surfaces of 12 teeth softened at a relatively slower rate of 5.8 per cent per hour. This difference was significant at $P < 0.01$.

Hardness of Naturally Carious Areas.—The hardness of a number of naturally carious lesions was also measured. Although suitable areas were difficult to find, some measurements were made, usually on interproximal surfaces. Typical results are shown in Fig. 3 which gives a diagram of the hardness measurements across a carious surface. Similar hardness measurements were also obtained when cavitation was evident. Such areas had hardness values usually falling in the range 100 to 250 K. N. This range corresponds to that for ground enamel surfaces after exposure to the buffer for about 8 to 10 hours.

Integrity of Enamel Surface During the Softening Process.—In order to determine whether appreciable loss of surface material occurred during the softening process produced by the buffer, the original indentations were remeasured after the 8-hour period. The lengths of the indentations showed no significant decrease (corresponding to less than 1.5 μ in length or 6 K. N.) although the teeth had dropped in hardness to the range 125 to 200 K. N.

DISCUSSION

The terms "hard" and "soft" teeth have been used frequently in the dental literature.[9] Pickerill[10] formulated an empirical classification of sclerotic teeth, characterized by hardness and immunity to caries, and malacotic teeth, characterized by comparative softness and susceptibility to caries. In order to determine whether the data of the present work would indicate a correlation between initial hardness and resistance to softening, the initial hardness was plotted against the rate of softening for each tooth at each time period and the regression lines calculated.

The equations were: $x = 7.9 \times 10^{-3}y + 365$ and $y = 2.9 \times 10^{-5}x + 21$ (x = initial hardness) (y = decrease in K. N. per hour) and the corresponding lines were almost perpendicular to each other. As the coefficient of correlation was practically zero ($r = 2.8 \times 10^{-4}$), the initial hardness showed no

relation to the rate of decrease of hardness. This finding was similar to that reported by Swartz and Phillips[11] who found no correlation between the initial hardness of ground surfaces and the amount of phosphorus dissolved by a buffer in a standard time interval, and to the earlier studies of W. D. Miller.[9]

The marked tendency for the slopes of the lines for the individual teeth to be parallel (Figs. 1 and 2) also indicates that the rate of softening is independent of the initial hardness. However, although the rates seem to be constant and similar, the harder teeth tend to take longer to reach any arbitrary extent of softening. In this sense then, hard teeth are more resistant than soft teeth.

The intact tooth surfaces were shown to soften at slower rates than ground surfaces. This result is probably related to the earlier observations that intact surfaces are less soluble than ground surfaces.[12, 13] Since the surface layers have been shown to have a high fluoride content and fluoride-treated enamel is less soluble than normal enamel, the lesser rate of softening of the intact surface may arise from the fluoride effect.

The outer enamel layer not only has a much higher concentration of fluoride than the underlying enamel but also appears to accumulate fluoride with age.[14, 15] Teeth used in the present experiments were human anteriors from persons usually older than 30 years and, hence, would be expected to have accumulated a considerable quantity of fluoride in the surface layer. This accumulation of fluoride may explain the present results (Table II) that a fluoride treatment had no effect on intact surfaces. Previous studies[16, 17] using radioactive fluoride indicated that the uptake of fluoride is greatest at ground or etched surfaces or in defective areas. As might be predicted from these studies, the results of the present work showed a significant decrease in the rate of softening of ground enamel which had been treated with fluoride.

The absence of an enhanced resistance of intact enamel surfaces after fluoride treatment as found in the present work may seem inconsistent with the known efficacy of topical fluoride treatments in vivo. However, topical treatments are usually made on young teeth, and the teeth in the present work were relatively old. In addition, any enhancement of resistance in adult teeth may be highly localized, since extensive variations of hardness are found in single teeth.[4] These soft "spots" presumably will take up fluoride better than harder areas. Although the resistance of these "spots" to solution may be increased by uptake of fluoride, the increased resistance might not show up in the present measurements which average the effect over a considerable area. Such soft "spots" are present in unerupted teeth[4] and presumably represent defective or incomplete development or calcification.

SUMMARY

Human incisor teeth were exposed to 0.001 M lactate buffer at pH 5.0 and 35° C. The rate of softening of the enamel was measured by microhardness tests for exposure times up to 10 hours. A linear rate of softening was

found and the softening could be measured as per cent drop of hardness per hour. Cows' teeth gave similar results but the rate of softening was somewhat greater than for human teeth.

Intact enamel surfaces were found to soften at a slower rate than ground surfaces, and the rates were closely similar for all teeth studied. The rate of softening was independent of the initial hardness. Since hard teeth took longer to soften to a given hardness, hard teeth may be considered to be more resistant than soft teeth.

Prior exposure of enamel to fluoride solution reduced the rate of softening of ground surfaces but not of intact surfaces.

The results suggest that the effects of buffers are similar to true carious attack on enamel.

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THE EFFECT OF LOCAL X-RAY IRRADIATION UPON THE TEETH OF THE ADULT AXOLOTL (SIREDON MEXICANUM)

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DESPITE great differences between mammals and urodele amphibians, the effects of x-rays upon these two groups are similar (Brunst, Sheremetieva-Brunst, and Figge[1]). Because their tooth development is also similar, an investigation of the effect of x-rays upon the teeth of urodele amphibians is of interest also in human stomatology.

In a previous investigation, Brunst, Sheremetieva-Brunst, and Figge[2] found that in young axolotls (the larval form of the Mexican salamander—*Siredon mexicanum*) the mechanisms of tooth formation and tooth maintenance are permanently destroyed by local x-ray irradiation.

The importance of age as a factor in the radiosensitivity of animals is a well-established fact. In general, the older the organism, the less its radiosensitivity. The radiosensitivity of young axolotls was investigated by Sheremetieva.[3] According to this author, 150 r is the lethal dose for freshly hatched axolotls, all of which died within one year; however, some animals irradiated with this dose 6 days after hatching lived 3 years. The lethal dose for 100 per cent of the animals irradiated 20 days after hatching is 300 r, and for animals irradiated 30 days after hatching it is 450 r.

An investigation of the x-ray sensitivity of adult axolotls (Brunst[4]) showed that their mean survival time after total body irradiation was as follows: after 6,000 r, 28 days; after 3,000 r, 44 days; after 1,000 r, 55 days. A dose of 500 r killed about 50 per cent of the animals; their mean survival time was 196 days; the remainder recovered, and some lived more than 2 years after irradiation.

An investigation of the effect of x-rays on the head of young axolotls (Brunst[5]) showed that the dose of 3,000 r was lethal in all cases if the whole head was irradiated (all animals died within 6 months after treatment). However, if only the anterior portion of the head, back to and including the eyes, was irradiated with 3,000 r, some animals lived more than 20 months.

The purpose of this work was to investigate the effects of local x-ray irradiation on the teeth of the adult axolotl.

MATERIAL AND METHODS

Thirty adult axolotls, 4 to 5 years old, were used. Ten animals served as untreated controls. The anterior portion of the head of 20 animals was irradiated with 6,000 r. Irradiation conditions were: 250 kv., 30 Ma., 0.25 mm.

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copper filter, beryllium window tube, half value layer 0.95 mm. copper, intensity 685 r/min. in air; target-object distance, 25 cm. During irradiation, the animals were not anesthetized, but were restrained by means of elastic bandages and arranged in radial positions on a circle of moistened filter paper. The nonirradiated portions were covered with moist cotton and shielded by lead covering with a round radiation localizer about 10 cm. in diameter (Brunst[6]). Fixations using acetic mercuric chloride formalin (Stieve) were made 6 to 150 days after treatment. The material was embedded in paraffin with 5 per cent beeswax, and sectioned at 8 or 10 μ. The sections were stained with Ehrlich's hematoxylin and eosin stain. Photomicrographs were taken at several different magnifications.

RESULTS

Normal Teeth of Adult Axolotl.—Two types of teeth are found in adult axolotl: (a) teeth in the anterior portion of the jaws corresponding to the incisors of higher animals, and (b) teeth in the posterior portion of the jaws corresponding to the molars of higher animals.

Teeth of the first group (Fig. 1) have an elongated form and are always connected with bone. Those of the second group (Fig. 2) are usually wider and a little shorter and are not always connected with bone; they may be located in the loose connective tissue under the oral epithelium. In a normal tooth, only the upper portion is in close contact with the oral epithelium and the root is embedded in bone or in contact with loose connective tissue through the dental pulp (Figs. 1 and 2).

In adult animals new enamel organs are continuously formed in the oral epithelium (Figs. 1 and 2). Contact of the teeth with bone is observed much later.

The Teeth of Irradiated Adult Axolotl.—The teeth of animals sacrificed during the first 50 days after irradiation appear quite different from those of animals sacrificed later. In the first group the teeth usually appear normal; they are comparatively large, in correct position, and are connected with bone (Fig. 6). These teeth were formed before irradiation. Teeth formed after irradiation are in an abnormal position and are completely isolated from the bone (Fig. 5). Such teeth were rarely found in this group.

In animals sacrificed later than 50 days after irradiation, most teeth are abnormal. Rarely found are teeth only slightly abnormal in structure and position (Figs. 3 and 4) showing a tendency to penetrate deeply into the oral epithelium and lose connection with loose connective tissue or bone. Irradiated teeth (Figs. 3 and 4), when compared with normal young teeth of a control animal (Fig. 2), show an abnormally thick epithelial layer (*EL*) which isolates the dental pulp from the loose connective tissue. Some of these teeth even have an abnormally oblique position (Fig. 3). In other irradiated animals, teeth are completely isolated in oral epithelium. Often they are horizontal and parallel to the outer surface of oral epithelium (Figs. 5, 7, and 8). Only a few teeth are in normal positions (Fig. 7, *E* and *F*).

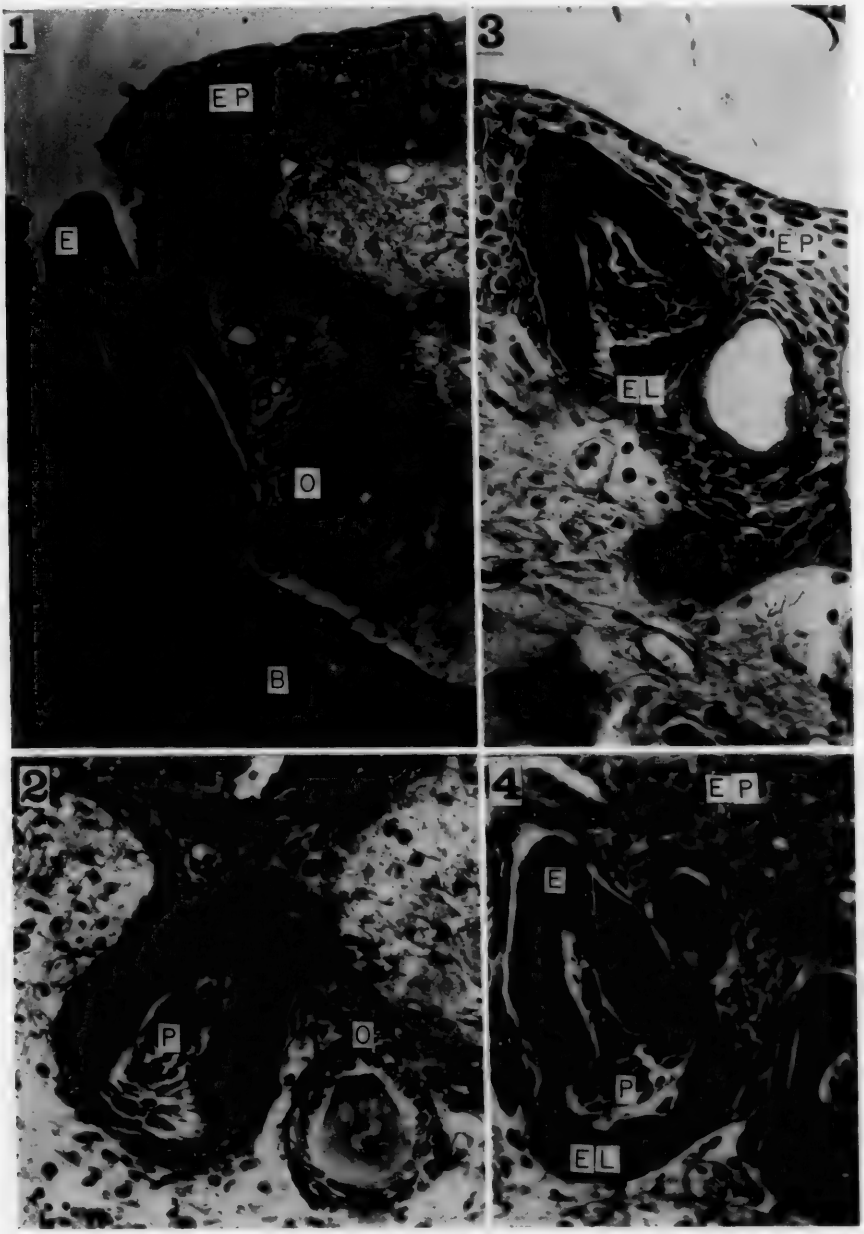
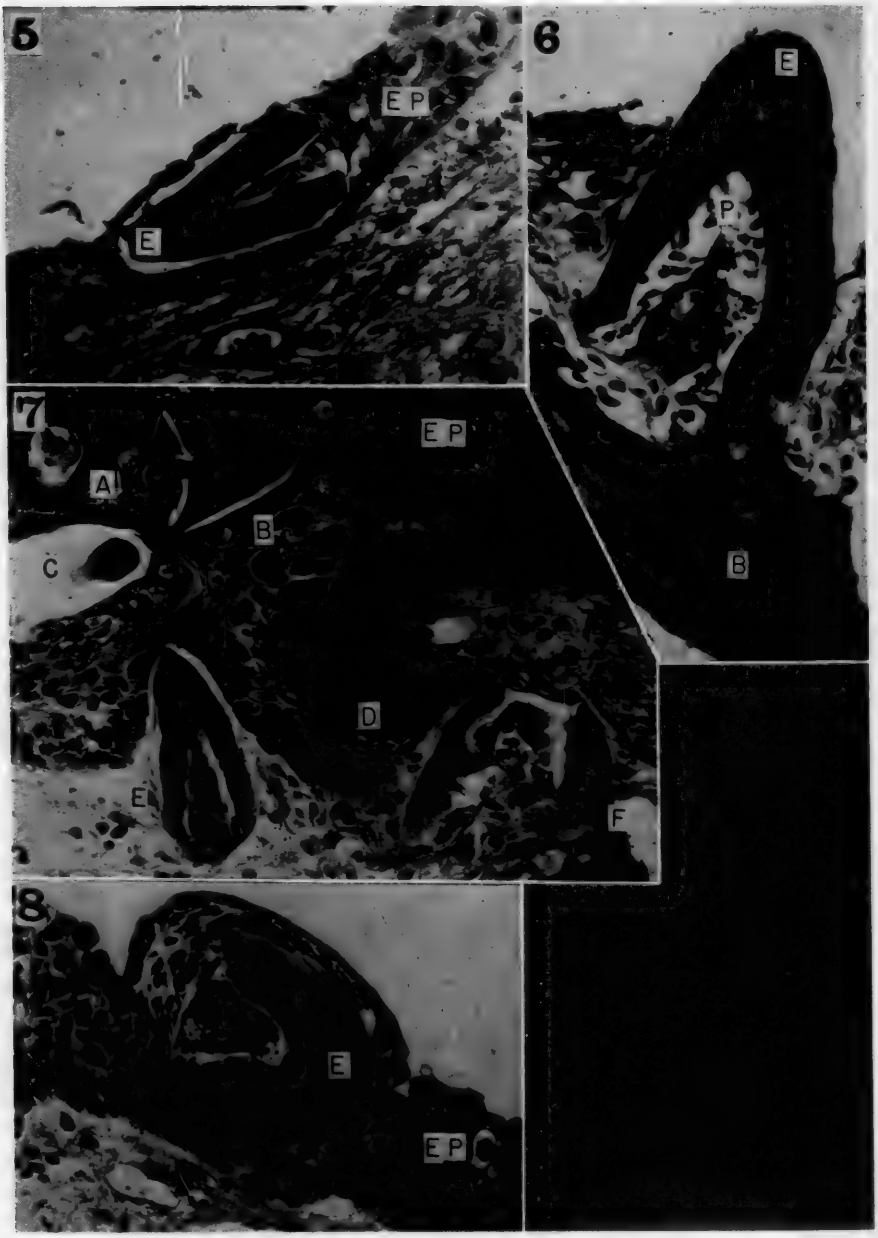


Fig. 1.—A sagittal section through the anterior portion of jaw of adult control axolotl. *B*, bone; *E*, enamel of the tooth; *EP*, oral epithelium; *O*, formation of new enamel organ. (Orig. mag. ×160.)

Fig. 2.—A sagittal section through the posterior portion of jaw of adult control axolotl. *O*, formation of new enamel organ; *P*, dental pulp. (Orig. mag. ×160.)

Fig. 3.—A sagittal section through the jaw of adult axolotl (No. 18) 89 days after irradiation of the anterior portion of the head with 6,000 r. *EP*, oral epithelium; *EL*, epithelial layer between the root and connective tissue. (Orig. mag. ×160.)

Fig. 4.—Another area of the sagittal section through the jaw of the same animal (No. 18) as that in Fig. 3. *E*, enamel of the tooth; *EP*, oral epithelium; *EL*, epithelial layer between the root and connective tissue; *P*, dental pulp. (Orig. mag. ×160.)



Figs. 5 and 6.—Various areas of sagittal sections through the jaw of an adult axolotl (No. 4) 43 days after irradiation of anterior portion of the head with 6,000 r. *B*, bone; *E*, enamel of the tooth; *EP*, oral epithelium. (Orig. mag. ×160.)

Fig. 7.—A sagittal section through the jaw of an adult axolotl (No. 15) 90 days after irradiation of anterior portion of the head with 6,000 r. *A*, *B*, *C*, *D*, *E*, and *F*—teeth in different position; *EP*, oral epithelium. (Orig. mag. ×160.)

Fig. 8.—A sagittal section through the anterior portion of the jaw of an adult axolotl (No. 18) 89 days after irradiation of anterior portion of the head with 6,000 r. *E*, enamel of the tooth; *EP*, oral epithelium. (Orig. mag. ×160.)

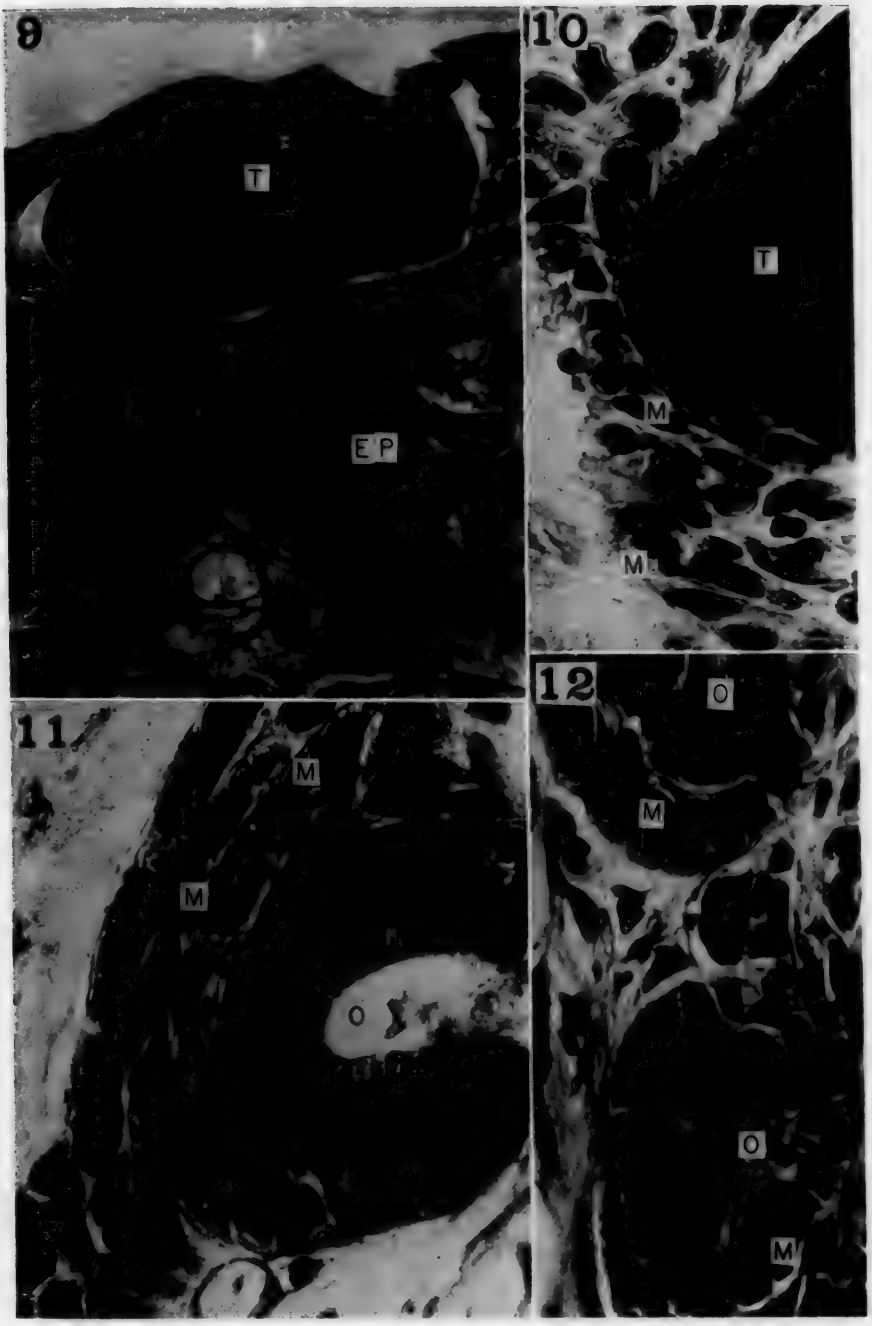


Fig. 9.—*A* sagittal section through the jaw of an adult axolotl (No. 10) 146 days after irradiation of anterior portion of the head with 6,000 r. *EP*, oral epithelium; *T*, tooth. (Orig. mag. ×500.)

Fig. 10.—A sagittal section through the jaw of an adult axolotl (No. 18) 89 days after irradiation of anterior portion of the head with 6,000 r. Mitotic activity in oral epithelium near the tooth. *T*, tooth; *M*, mitotic figure. (Orig. mag. ×500.)

Figs. 11 and 12.—Sagittal sections through the jaws of adult axolotls (Nos. 18 and 7) 89 and 91 days after irradiation of anterior portion of the head with 6,000 r. Mitotic activity during formation of new enamel organs, *O*; *M*, mitotic figures. (Orig. mag. ×500.)

The dental pulp in some cases is completely isolated in surrounding oral epithelium (Figs. 5 and 8), or completely absent (Fig. 9). Isolation of the teeth in oral epithelium and disappearance of dental pulp are facts being established in this investigation. The study was made on serial sections and, when the teeth were sectioned longitudinally and all pulp was clearly seen (Figs. 5 and 8), no connection was found between isolated pulp and subepithelial loose connective tissue. Also, in some teeth that were sectioned longitudinally, dental pulp was totally absent (Figs. 9 and 14, *A*). Comparison of Fig. 1 and Fig. 8 shows the extent of difference between teeth in the anterior portion of the jaw of control and irradiated animals. It is interesting that even the neighboring teeth in an irradiated animal can be oriented in opposite directions (Figs. 13 and 14). Fully developed teeth, which are in irregular position, are usually accompanied by many enamel organs (Fig. 14). In some such cases, a very long epithelial outgrowth penetrating the loose connective tissue with enamel organs for 7 or 8 teeth is observed. The formation of so many enamel organs in adult animals is an abnormal phenomenon.

DISCUSSION

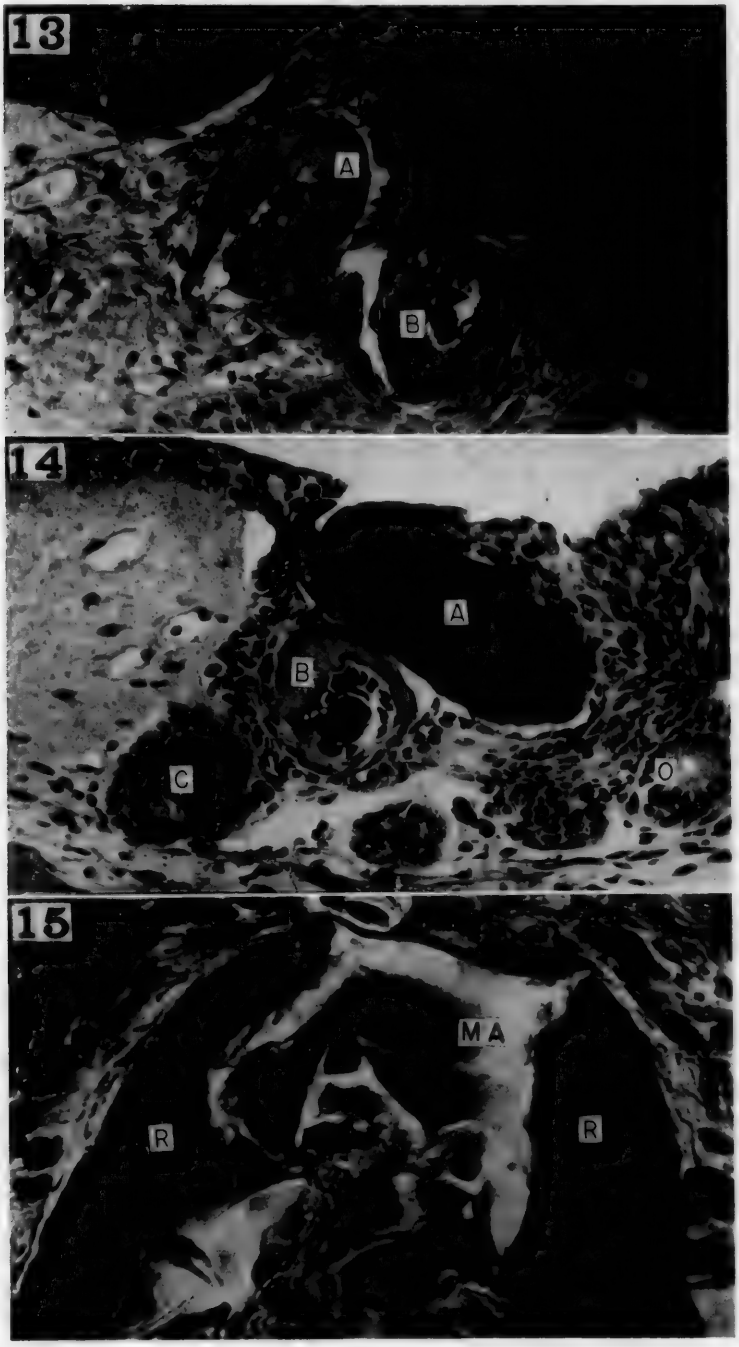
Comparison of the teeth in control and irradiated animals indicates that their response to irradiation follows a characteristic pattern.

During the first 50 days after irradiation, only the normal teeth formed before irradiation are usually found. At the end of this period, they begin to disappear. The process of disappearance of teeth probably takes place quickly and is usually complete within 10 to 15 days. Only rarely are remnants of old teeth present 90 days after irradiation (Figs. 7, *E*, 15, and 18). These cases are especially important because they show clearly that the disappearance of teeth may be the result of activity by giant cell macrophages. The crown portion of the teeth is destroyed and very large macrophages penetrate the dental pulp (Figs. 15 and 18). The role of macrophages in the destruction of irradiated tissues has been observed many times in earlier investigations (Brunst, Sheremetieva-Brunst, and Figge,[2] Brunst[7-9]). Tooth substance is probably dissolved by lytic enzymes produced by the macrophages (Brunst[9]). Simultaneous with the disappearance of old teeth is the formation of new teeth. These new teeth are usually much smaller, more irregular in shape and in position. They are never connected with the bone and only occasionally with connective tissue. The presence of an unusual number of enamel organs (sometimes for 7 or 8 teeth) and the presence of many mitotic figures in the oral epithelium (Fig. 10), especially in young enamel organs (Figs. 11 and 12), indicate that these are newly formed teeth.

Fig. 13.—A sagittal section through the jaw of an adult axolotl (No. 15) 90 days after irradiation of anterior portion of the head with 6,000 r. *A* and *B*, teeth in different positions. (Orig. mag. ×160.)

Fig. 14.—A sagittal section through the jaw of an adult axolotl (No. 7) 91 days after irradiation of anterior portion of the head with 6,000 r. *A*, *B*, and *C*, teeth in different position; *O*, formation of new enamel organ. (Orig. mag. ×160.)

Fig. 15.—A sagittal section through the jaw of an adult axolotl (No. 15) 90 days after irradiation with 6,000 r. *MA*, giant macrophage; *R*, remnants of the tooth wall. (Orig. mag. ×500.)



Figs. 13-15.—*For legends see opposite page.*

The entire absence of pulp in many teeth completely surrounded with oral epithelium was observed. This was unexpected and may be a secondary phenomenon and some causes of disappearance of the pulp must exist. It is clear that isolation of teeth in oral epithelium creates unfavorable conditions for existence of the dental pulp. In a tooth completely surrounded with avascular epithelium, the pulp mesenchyme would probably suffer from a deficiency of oxygen and ischemic necrosis may develop. As a result of such necrosis, the pulp may disappear completely.

How is a new generation of teeth formed after destruction of the old one in irradiated jaws? The irradiation dose may be insufficient to destroy totally the ability of oral epithelium to form new enamel organs. The possibility of x-ray stimulation by disintegration products is doubtful but cannot be ignored (Brunst[9, 10]).

How long can the new generation of teeth exist? The majority are unable to function and they gradually disappear. In some animals, fixed about 150 days after irradiation, almost all teeth disappear and only occasional small and completely abnormal teeth are found (Fig. 9). Figs. 16 and 18 are photomicrographs showing macrophages under the root of a tooth in an animal fixed 145 days after irradiation. This tooth is comparatively large and appears normal but is obviously in the process of being destroyed by macrophages from the basal end. Therefore, we conclude that teeth formed after irradiation gradually disappear as a result of the activity of macrophages. Later fixation would probably show that the animals are completely without teeth. But this problem requires additional investigation.

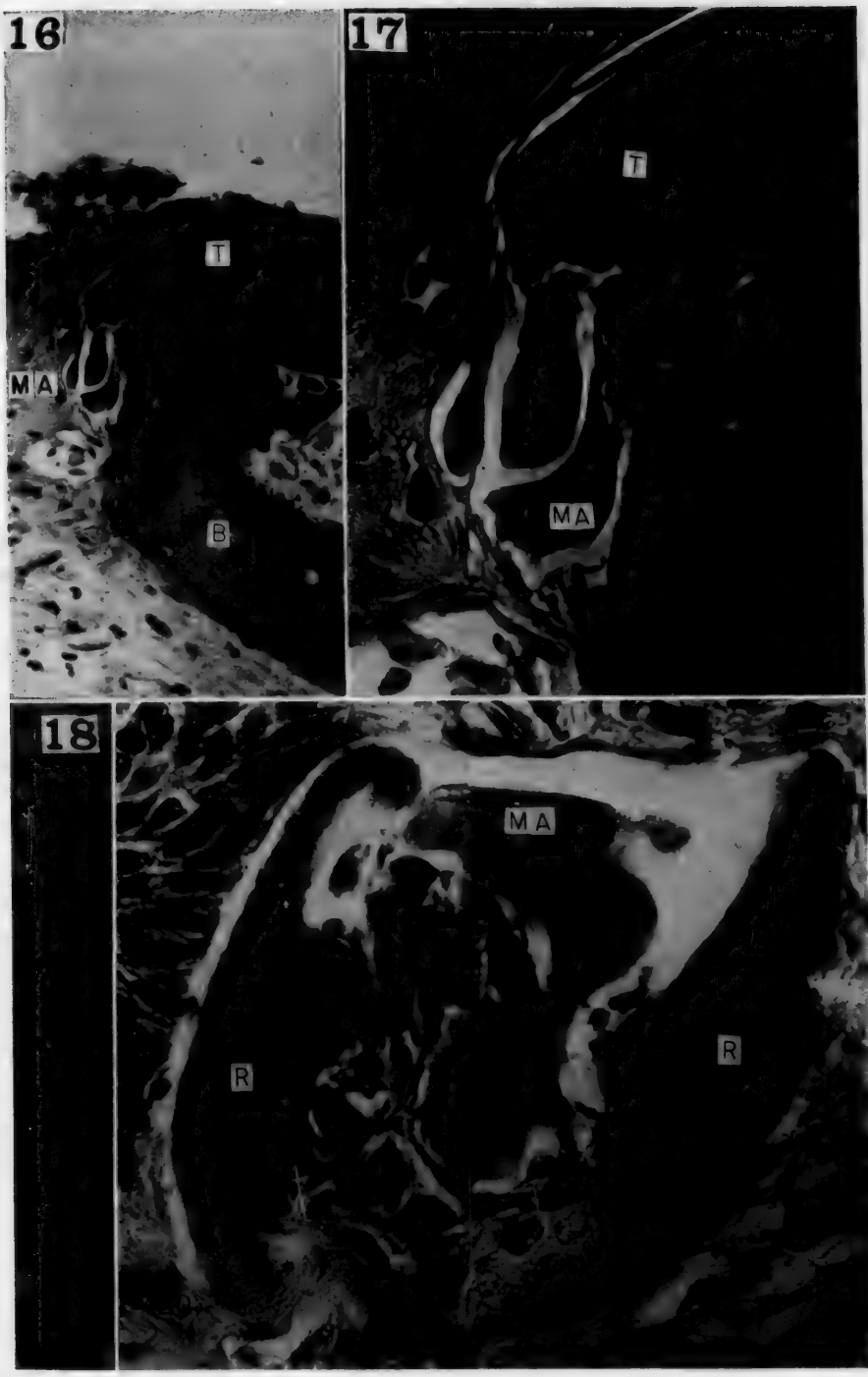
Although the mechanism of tooth formation and tooth maintenance is permanently destroyed in young animals by x-ray irradiation of 2,000 to 4,000 r, this effect is not observed in adult animals after much larger doses (6,000 r). In adult animals, the mechanism of tooth formation, although greatly damaged, is not destroyed and a new generation of abnormal teeth is formed. These teeth also tend to disappear about 150 days after treatment as a result of the activity of macrophages.

SUMMARY

The anterior portion of the head, back to and including the eyes, of 20 adult axolotls (*Siredon mexicanum*) was irradiated with 6,000 r. During the first 50 days after treatment, only normal teeth were observed. At the end of this period they began to disappear. This process usually took place quickly within 10 to 15 days. The disappearance of teeth was a result of the activity of giant cell macrophages. Along with the disappearance of old teeth, a new generation of teeth began to form. These teeth were much smaller, irregular in shape and in position. They were never connected with the bone and only

Figs. 16 and 17.—A sagittal section through the jaw of an adult axolotl (No. 17) 144 days after irradiation with 6,000 r. The same place of section under various magnifications. *B*, bone; *T*, tooth; *MA*, macrophages. (Orig. mag. ×160 [Fig. 16] and ×500 [Fig 17].)

Fig. 18.—A sagittal section through the jaw of an adult axolotl (No. 15) 90 days after irradiation with 6,000 r. *MA*, giant macrophage; *R*, remnants of the tooth wall. (Orig. mag. ×500.)



Figs. 16-18.—*For legends see opposite page.*

in some cases with connective tissue. These new teeth, usually unable to function, also gradually disappeared. In some animals, fixed about 150 days after irradiation, almost all teeth had disappeared and only an occasional small tooth could be found. The presence of macrophages under the root of some teeth in an animal fixed about the same time showed that the new generation of teeth also gradually disappeared as a result of the activity of macrophages.

The mechanism of tooth formation and tooth maintenance in the adult axolotl was greatly damaged by irradiation of 6,000 r.

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A SELECTIVE MEDIUM FOR COUNTING FUSIFORM ORGANISMS IN DENTAL SPECIMENS

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FUSOBACTERIA are gram-negative, anaerobic rods with tapered ends. In artificial media, they often show the pleomorphic L-morphology.[1] They are found in large numbers in association with oral spirochetes, in necrotizing ulcerative gingivitis, and in pathologic conditions of the gingiva and oral mucosa. They are present along with many other kinds of gram-negative microorganisms (Rosebury, MacDonald, and Clark,[2] Schultz-Haudt, Bruce, and Bibby[3]). The occurrence of fusobacteria in the mouth has been discussed in relationship to their possible etiological role in oral infection. Bøe[4] showed that fusobacteria alone, without the cooperation of other microbes, can be pathogenic for human beings. Despite this fact, it is recognized that these same organisms are also found in healthy mouths, and we have observed them in the edentulous mouth of a healthy 2-month-old infant. Counting these organisms in healthy and diseased mouths may open another approach to understanding the role of these indigenous organisms in the mouth. Development of a new selective medium for counting colonies of these organisms is greatly needed.

It is generally believed that a medium for these organisms should be enriched with protein-rich animal fluids, glucose, cysteine, vegetable extract,[5] and yeast extract.[6] (An alkaline medium[7] also gave good growth.) These substances, however, are known to be beneficial not only for the growth of fusiform bacteria but also for numerous other bacteria. Selective media should contain substances which inhibit the growth of the other contaminants and which do not harm those organisms under study. The bacteriostatic action of the dyes, crystal violet, gentian violet, and brilliant green, was utilized by Slanetz and Rettger[5] and by Bøe[4] for this purpose. Omata[8] found that fusobacteria resisted the antibiotic action of streptomycin.

EXPERIMENTS AND RESULTS

This study consists of four parts: (1) determination of a basal medium, (2) development of a selective medium, (3) enrichment of the selective medium, and (4) examination of the effect of the selective medium.

The major part of the material in this paper was presented at the 29th General Meeting of the Japan Bacteriological Society, April 4, 1956.

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TABLE I

THE DISAPPEARANCE OF FUSIFORM ORGANISMS IN A BROTH ENRICHED WITH GLUCOSE

	MEDIA		DONORS OF DENTAL PLAQUE MATERIAL										AVERAGE
	INITIAL pH	GLUCOSE CONTENT (%)	O. K.	O. M.	S. H.	F. U.	H. A.	I. T.	S. H.	S. E.	N. A.	M. A.	pH
1.	8.0	0	7.6† F St V Ae	7.6 F St V Ae	7.8 F St V Ae	7.8 F St V Ae	7.8 F St V Ae	7.6 F St V Ae	7.2 F St V Ae	7.8 F St V Ae	7.8 F St V Ae	7.6 F St V Ae	7.7
2	8.0	0.1	7.2 F St V Ae	7.0 F St V Ae	7.4 F St V Ae	7.2 F St V Ae	7.0 F St V Ae	6.2 F St V Ae	6.4 F St V Ae	7.4 F St A Ve	7.2 F St V Ae	7.0 F St V Ae	7.0
3.	8.0	0.2	6.8 F St V Ae	6.8 F St V Ae	7.0 F St V Ae	6.8 F St V Ae	6.6 F St V Ae	6.2 F St V Ae	6.0 F St V Ae	6.8 F St V Ae	6.6 F St V Ae	6.2 F St V Ae	6.6
4.	7.0	0.1	6.6 F St V Ae	7.2 F St V Ae	6.0 F St V Ae	6.6 F St V Ae	6.4 F St V Ae	6.8 F St V Ae	6.6 F St V Ae	6.6 F St V Ae	6.3 F St V Ae	6.4 F St V Ae	6.6
5.	6.0	0.1	6.0 F St V *	5.8 * St V *	5.8 F St V *	5.8 * St V *	5.8 * St V *	5.2 * St V *	5.6 * St V *	5.6 * St V *	5.8 * St V *	5.8 F St V Ae	5.7
6.	8.0	0.4	6.2 F St V Ae	6.2 F St V Ae	6.4 * St V *	6.8 * St V *	4.4 * St V *	4.4 * St V *	4.4 * St V *	5.4 * St V *	5.8 * St V *	4.3 * St V *	5.4
7.	7.0	0.2	5.8 F St V Ae	4.8 F St V *	5.8 * St V *	5.8 * St V *	5.8 * St V *	5.0 * St V *	5.6 * St V *	5.6 * St V *	4.8 * St V *	5.4 * St V *	5.4

8.	8.0	0.8	4.8 * St V *	4.6 * St V *	5.6 * St V *	4.4 F St V *	4.2 * St * *	4.4 * St * *	4.3 * St * *	4.2 * St V *	4.2 * St * *	4.2 * St * *	4.5
9.	7.0	0.4	4.0 F St V *	4.2 F St V *	4.4 * St V *	4.8 * St V *	4.2 * St V *	4.0 * St V *	4.0 * St V *	4.4 * St V *	4.0 * St * *	4.0 * St * *	4.2
10.	7.0	0.8	4.0 F St V *	4.2 * St * *	4.4 * St V *	4.2 * St V *	4.2 F St V *	4.0 * St * *	4.4 * St V *	4.0 * St * *	4.7 * St * *	4.4 * St * *	4.3
11.	6.0	0.2	4.2 F St V *	4.2 * St V *	4.4 * St V *	4.4 * St V *	4.4 * St V *	4.6 * St V *	4.2 * St V *	4.6 * St * *	4.2 * St V *	4.2 * St * *	4.3
12.	6.0	0.4	4.0 * St V *	4.0 * St V *	4.4 * St * *	4.3 * St V *	4.0 * St V *	4.0 * St V *	4.3 * St * *	4.0 * St * *	4.0 * St * *	4.0 * St V *	4.1
13.	6.0	0.8	4.0 F St V *	4.2 * St * *	4.4 F St * *	4.4 * St V *	4.4 * St V *	4.0 * St * *	4.0 * St * *	4.0 * St * *	4.0 * St * *	4.4 * St * *	4.2

F—Fusiform organisms.
St—Streptococci.
V—Small gram-negative cocci.
Ac—Marginal growth.
*—Absence of either one of these.
†—These figures are the pH of the media after 2 days' culture.

1. *Determination of a Basal Medium.—*

Influence of glucose in the basal medium: Thirteen different media were prepared utilizing different levels of initial pH and glucose concentration. These consisted of 1 per cent polypeptone, 1 per cent Ehrlich meat extract, 0.25 per cent NaCl, and glucose, as shown in Table I. Each medium was inoculated separately with dental plaque material from 10 donors. Morphologic findings and pH decreases in the course of 2 days' incubation at 37° C. were variable, but there was evidence that increased glucose content caused a lower pH which, in turn, seemed to influence the disappearance of fusiform organisms. They were found in broths with an average pH of 6.6 (6.0 to 7.2) where they produced a disagreeable odor. In the media in which the pH had decreased to lower than 6.0, streptococci predominated and there was no odor. In a few media in which the pH fell to 4.0, a very small number of gram-negative, large, filamentous forms, presumably fusiform organisms, were found among the streptococci. In alkaline broth at pH 8.0, fusiform organisms grew in all cases, but they were always accompanied by small gram-negative cocci (presumably Veillonella), large gram-negative cocci (mainly Neisseria), and streptococci. In addition, gram-positive diphtheroids, micrococci, gram-negative rods, comma shapes, and bacteroides were found at times. Neisseria were present in a bacterial pellicle formed on the surface of the broth. Due to the known multiplicity of the oral flora, these limited findings may indicate that alkaline broth permitted only limited bacterial growth.

Alkaline broth tubes were inoculated with 222 specimens from 124 persons of various ages. Table II shows that this medium detected fusiform organisms in the specimens with almost complete regularity. In the following studies, alkaline broth tubes were used both for subculturing as well as for the initial basal medium.

TABLE II
INCIDENCE OF FUSIFORM ORGANISMS IN HUMAN MOUTHS
(ALL SPECIMENS ENRICHED IN ALKALINE BROTH)

SOURCE OF SPECIMENS		NUMBER OF EXAMINATIONS	NUMBER OF POSITIVE CULTURES
1. 67 adults (56 males and 11 females)		165	165
2. 54 children, aged from 4 to 6 years of age (27 males and 27 females)		54	53
3. 3 infants—2, 14, and 16 months of age		3	3
Totals	124	222	221
Incidence	99.2%		

2. *Development of the Selective Medium.—*

Subcultures prepared in alkaline broth were made from specimens from 10 different donors in order to observe the individual differences. A loopful from each subculture was transferred into a trial medium which was composed of the basic alkaline broth with the addition of the other substances. After incubation, the selective effect of the medium was evaluated by examining gram-stained slides prepared from the culture.

Elimination of aerobic organisms: Overlaying of petroleum paraffin on the surface of the alkaline broth eliminated marginal growth well, but it was impractical because oiliness made further investigation difficult. The addition of sodium thioglycolate to the basal broth did not have any conspicuous advantage. Addition of 1 per cent or more of sodium azide inhibited all growth. Soduim azide, 0.02 to 0.01 per cent, was toxic for the growth of large gram-negative cocci, gram-negative rods, micrococci and diphtheroids, and also greatly reduced the growth of small gram-negative cocci. Thus, 0.01 per cent sodium azide alkaline broth permits the growth mainly of fusiform organisms, streptococci, and small numbers of small gram-negative cocci.

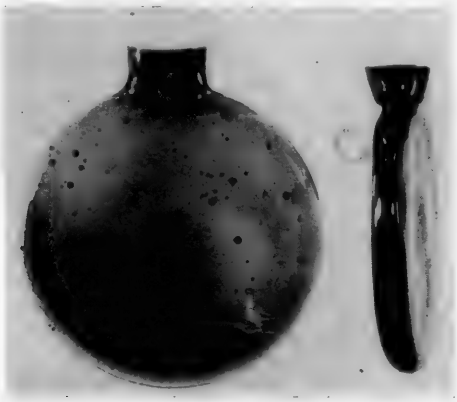

Fig. 1.—Banjo-shaped flask.

Elimination of streptococci and small gram-negative cocci: The concentration of crystal violet, generally recommended for agar media, was so effective in the azide alkaline broth that no regular growth was obtainable and, even in a broth containing crystal violet at a concentration of only 1:640,000, growth was greatly inhibited. Addition of agar even in a small amount (0.1 per cent) overcame this difficulty. Studies were then made to determine the minimum effective concentration of crystal violet in the azide alkaline semisolid medium. Even at dilutions of 1:80,000 to 1:40,000, no oral bacteria except fusiform organisms grew. In this medium, colonies of fusiform organisms took different shapes according to the concentration of agar (Fig. 2).

3. *Enrichment of the Selective Medium.*—

Glucose, some amino acids, and other growth stimulants were added to the selective medium. The effect of growth stimulants was estimated by counting colonies developed in a banjo-shaped flask (Fig. 1) containing the intermediate trial medium which had been inoculated with the same amount of subculture prepared in the alkaline broth.

In the presence of small amounts of agar, glucose did not aid the growth of streptococci but favored the growth of fusiform organisms (Table III and

Fig. 3). Both cysteine and cystine also stimulated the growth of the fusiform organisms, with the vigorous production of H_2S gas which reduced crystal violet to a colorless state (Table IV).

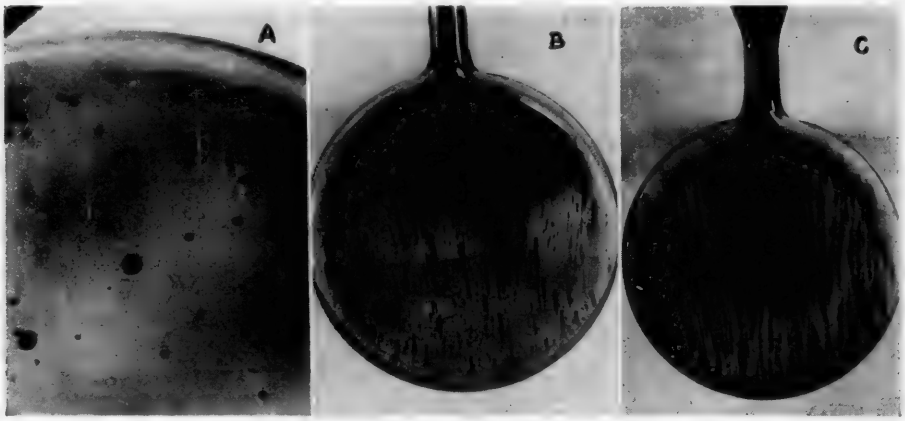

Fig. 2.—Colony shapes of fusiform organisms developed on the proposed selective medium with different agar concentration. *A*, 0.3 per cent; *B*, 0.05 per cent; *C*, less than 0.05 per cent.

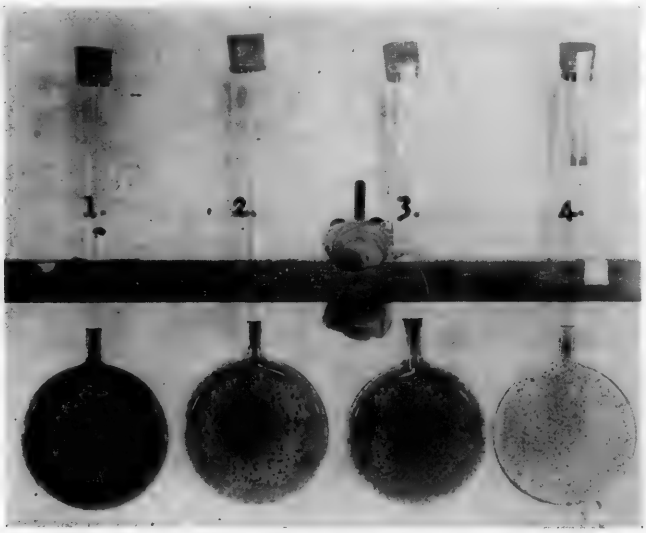

Fig. 3.—Enrichment of the basic selective medium by addition of glucose and cysteine. The numbers of flasks correspond to those of media in Table III.

Substitution of Japanese Ehrlich meat extract with freshly prepared meat extract, Difco meat extract, or Difco yeast extract did not improve the medium. Lowering of alkalinity of the medium to pH 7.5 did not result in any significant advantage. Serum, which is believed to be stimulatory to

TABLE III
ENRICHMENT OF THE BASIC SELECTIVE MEDIUM BY ADDITION OF GLUCOSE AND CYSTEINE OR CYSTINE

MEDIA	COUNT OF FUSIFORM ORGANISM IN FLASKS	AVERAGE
1. Basic selective medium	1	
	2	
	1	
	0	1
	1	
	0	
2. Enriched with 0.5 per cent glucose	686	
	586	
	286	359
	153	
	85	
3. Enriched with 1.0 per cent glucose	1,057	
	1,366	
	882	906
	730	
	494	
4. Enriched with 0.5 per cent glucose and 0.1 per cent cysteine	3,920	
	1,790	
	1,930	2,127
	2,090	
	906	
5. Enriched with 0.5 per cent glucose and 0.1 per cent cystine	2,420	
	1,940	
	2,520	2,246
	2,130	
	2,220	

The basic selective medium consists of polypeptone, 1 per cent; Ehrlich meat extract, 1 per cent; NaCl, 0.25 per cent; NaN_3, 0.01 per cent; agar, 0.3 per cent; crystal violet, 1:80,000; and pH, 8.0.

the growth of fusiform organisms, was added to the medium prepared by the final formula, but the turbidity of the serum obscured the clear view of colonies inside the flask.

Anaerobic atmosphere in phosphorus jars also provided favorable circumstances for the growth of these organisms when they were seeded on the surface of—and throughout—the selective medium, solidified by increasing agar content to 1.5 per cent.

4. *Examination of the Effect of the Selective Medium.*—

The final test of these experiments was carried out by inoculation of an oral specimen into the following medium at pH 8.0: polypeptone, 1 per cent; Ehrlich meat extract, 1 per cent; cystine, 0.05 per cent; sodium glutamate, 0.05 per cent; sodium chloride, 0.25 per cent; sodium azide, 0.01 per cent; crystal violet, 1:80,000; agar, 1.0 per cent (tryptophane was omitted here because of its expense). Oral microorganisms were obtained by rinsing the mouth with a peptone solution. Two-tenths or 0.5 ml. of the suspension was mixed in the melted selective medium in a test tube kept at approximately 45° C. After 3 days' incubation at 37° C., the medium was poured out into a sterile Petri dish and colonies were picked at random for the identification of their morphology.

TABLE IV
COMPARISON OF GROWTH-STIMULATING ACTION OF AMINO ACIDS

MEDIA	COUNT OF FUSIFORM ORGANISM IN FLASKS	AVERAGE	EFFECT OF ADDED AMINO ACID
1. Basic medium	262, 170, 176, 236, 215, 215, 202, 214	211	
2. With 0.05% l-histidine	254, 242, 290, 282	242	1.15
3. With 0.05% dl-threonine	166, 158, 180, 186	172	0.82
4. With 0.05% dl-glutamic acid	526, 588, 509, 589	553	2.62
5. With 0.05% dl-Serine	195, 184, 171, discarded	183	0.87
6. With 0.05% dl-Asparagine	252, 229, 236, 236	238	1.13
7. With 0.05% dl-tyrosine	180, 184, 191, 193	187	0.89
8. With 0.05% l-tryptophane	406, 350, 332, 306	349	1.65
9. With 0.05% dl-methionine	188, 165, 143, 159	164	0.78
10. With 0.05% dl-alanine	190, 179, 179, 145	173	0.82
11. With 0.05% glycine	158, 147, 162, 145	153	0.73
12. With 0.05% l-valine	104, 117, 129, 137	122	0.58

Basic medium consists of polypeptone, 1 per cent; Ehrlich meat extract, 1 per cent; NaCl, 0.25 per cent; glucose, 1 per cent; cysteine, 0.1 per cent; NaN_3, 0.01 per cent; crystal violet, 1:80,000; agar, 0.3 per cent; and pH, 8.0.

Six hundred and twenty colonies from 21 specimens taken from the 18 donors all consisted of cells of fusiform organisms, but 23 colonies of these (3.8 per cent) were contaminated by a few cells of either streptococci, gram-negative rods, gram-negative cocci, gram-negative commas, or yeast-like cells (Table V). Thus, a reasonably accurate estimate of fusiform organisms can be made by counting all colonies appearing on this selective medium.

Identification of fusiform organisms grown in the selective medium: Subcultures were prepared in the selective medium from 8 oral specimens from 7 donors and were plated on a glucose-blood-agar medium. Colonies of fusiform organisms were picked at random and purified by routine plating five times. Two hundred and twenty-nine isolates were tested in respect to H_2S and indol production, lead acetate paper strip and Kovac's reagent being applied, respectively. Acid production was tested with pH indicators.

On the basis of H_2S and indol production, 212 strains belonged to the proteolytic type and 4 strains to the saccharolytic type as described by Berger.[9] Thirteen strains were of a diverse type. Four saccharolytic strains,

TABLE V
IDENTIFICATION OF COLONIES GROWN IN THE SELECTIVE MEDIUM

NAME OF DONOR	NO. OF COLONIES DEVELOPED	NO. OF COLONIES TESTED	NO. OF COLONIES OF FUSIFORM ORGANISM	COLONIAL CONTAMINANTS
Michimura	More than 800	122	122	3 streptococci, 1 Candida, 1 gram-negative coccus, 1 gram-negative rod
Michimura	35	35	35	2 streptococci
Michimura	280	20	20	1 streptococcus
Kosuge	11	11	11	none
Kosuge	30	16	16	none
Ishizaki	More than 1,000	84	84	1 streptococcus
Ishii	40	40	40	none
Kato	101	20	20	none
Akita	150	19	19	none
Takizawa	14	14	14	1 gram-negative rod
Onisi	4	3	3	1 gram-negative rod
Wada	120	20	20	2 streptococci
Nakayama	232	22	22	1 streptococcus, 1 gram-negative comma, 1 gram-negative rod
Usami	420	32	32	1 gram-negative rod, 1 streptococcus
Ishiwara	More than 700	30	30	1 gram-negative coccus
Kagimoto	More than 1,000	29	29	1 streptococcus
Koike	More than 1,000	30	30	1 gram-negative comma
Takano	400	6	6	none
Kondo	47	26	26	none
Tachibana	600	26	26	1 gram-negative rod
Shitara	450	15	15	none
Totals		620	620	23 contaminated

TABLE VI
FERMENTATION CHARACTERISTICS AND H_2S AND INDOL PRODUCTION BY 229 FUSIFORM ORGANISMS GROWN IN THE NEW SELECTIVE MEDIUM

SUGAR FERMENTATION				PRODUCTION OF BOTH H_2S AND INDOL	PRODUCTION OF EITHER H_2S OR INDOL	PRODUCTION OF NEITHER H_2S NOR INDOL
GLUCOSE	SUCROSE	MALTOSE	LACTOSE			
X	–	–	–	104	3	1
X	X	–	–	48	3	3
X	X	X	X	0	1	0
–	–	–	–	0	1	0
Unknown*				60	5	0
Totals				212	13	4†

X—Acid from sugar.
*Died before the time of the fermentation test.
†Final pH's of these four strains were not lower than 6.0.

however, produced acid from neither maltose nor lactose and failed to decrease pH of the media containing glucose and sucrose to the extent reported by Bøe.[4]

DISCUSSION

Crystal violet was used for the elimination of gram-positive bacteria, mainly streptococci, in this new selective medium. Ingraham[10] and Hoffmann and Rahn[11] considered that crystal violet at a dilute concentration acted as a poison by altering the oxidation-reduction potential to a range unfavorable

for cell multiplication of gram-positive bacteria. Further, Ingraham[10] observed that gentian violet, which has been decolorized by either oxidation or reduction, is practically nontoxic. In the present study, fusiform organisms were found to be rather sensitive to this dye; they often failed to grow in a broth containing crystal violet, even at a concentration of 1:320,000, and they did not multiply unless they were either accompanied by growth of gram-negative rods which reduced the dye to a colorless state, or inoculated in a broth with such a high dilution of crystal violet that streptococci could grow. It was found, however, that colonies which appeared in the semisolid selective medium were usually surrounded by a reduced yellow zone associated with vigorous production of H_2S bubbles. It is possible that the reducing power of H_2S might have detoxified the crystal violet, with the aid of the selective action of agar in such way that it kept H_2S around the cells at a high enough concentration to initiate initial cell multiplication. For successful inoculation, it is suggested that all clumps of cells be broken up in the inoculum taken from the mouth. The value of this procedure has been confirmed by counts of fusiforms from saliva samples which are now being carried out in this department.

That small gram-negative cocci, presumed Veillonella, are also sensitive to crystal violet, may be explained to some extent by the early observation of Foubert and Douglas[12] on *Micrococcus lactilyticus*. These organisms were gram positive in the youngest phase of growth. Tachibana[13] found that oral H_2S-producing Veillonella required aerobic conditions for growth. In the new selective medium, therefore, crystal violet and sodium azide may work together against oral Veillonella.

The action of sodium azide becomes stronger under acid conditions. Thus, its action may be increased in the presence of glucose, the most probable source of acid in media. Addition of azide may be a useful way to inhibit growth of aerobic gram-negative contaminants.

When glucose was added to a plain alkaline broth, streptococci grew almost exclusively. Utilization of glucose by these bacteria may be faster than by fusiform organisms and this may change the environment to one unfavorable for fusiforms. The bacteriostatic action of crystal violet may prevent all nutrients, especially glucose, being assimilated by streptococci and, thus, leave them available to fusiform organisms which are also aided by the selective action of agar.

According to the recent trend in the classification of this genus, Group 1 of Spaulding and Rettger,[14] fusobacterium of Bøe,[4] butylic acid fermentation type of Jackins and Barker[6] and proteolytic type as described by Berger[9] are in the same category. Group 2 of Spaulding and Rettger, Leptotrichia of Bøe, lactic acid fermentation type of Jackins and Barker, and saccharolytic form of Berger make up another category. Diverse strains from the above 2 groups are also described by Morris[15] and by Omata and Disraely.[16] However, because of lack of experiments on pure strains of oral gram-negative filamentous forms, classification of the saccharolytic form is still under discussion.

Recently, Kasai,[17] with oral filamentous forms, and Hamilton and Zahler,[18] with *Leptotrichia buccalis*, claimed difficulties of isolation and maintenance of isolates. Determination of biochemical activities of isolates should be made only where there is evidence of growth in a test medium, because even proteolytic forms will often stop growing in a broth for unknown reasons. The present experiment used semisolid media which became turbid after bacterial growth. Four strains which showed good growth and did not produce H_2S and indol were thought to be saccharolytic forms; however, their fermentation pattern did not coincide with that of saccharolytic forms,[10, 18] and they did not increase in pH as far as did the Leptotrichia reported in the literature.[4, 18] Also, they were maintained by transfer in a new artificial medium without any difficulties. These experiences seemed to indicate that they are not Leptotrichia but a diverse type of fusobacteria. At present, it would be better to withhold the final discussion on the identification problem until further studies are made. There are many problems involved here, including the difficulty of isolation, because of the extreme delicacy of this microorganism, its infrequent occurrence in the mouth, and the presence of some harmful ingredients in the present media.

In every respect, it can be said that the new medium is selective for the growth of fusobacteria as defined by Bøe, and also that the degree of selectivity is adequate for the estimation of the relative numbers of this type of fusiform organism in an oral specimen.

Use of the banjo-shaped flask is convenient in that it provides an anaerobic environment for the culture. This advantage outweighs the fact that fewer colonies grow in the flask than in the cultures carried out in Peri dishes containing the present medium with 1 per cent agar under strict anaerobic conditions.

SUMMARY

1. In order to obtain information about the role fusiform organisms play in a human mouth, quantitative measurements of these organisms were made on a selective medium.

2. This selective medium was composed of 1 per cent polypeptone, 1 per cent Ehrlich meat extract, 1 per cent glucose, 0.05 per cent cystine, 0.05 per cent sodium glutamate, 0.25 per cent sodium chloride, 0.01 per cent sodium azide, 1:80,000 crystal violet, and 0.3 per cent agar at pH 8.0. Use of a banjo-shaped culture flask was recommended for counting colonies.

3. Inclusion of a small amount of agar was important in allowing growth of fusiforms in the presence of glucose and crystal violet. Three-tenths per cent agar was used when culture was made in the flask, and 1.0 per cent agar when the culture was carried out in a Petri dish under strict anaerobic conditions. This latter method of culture produces more colonies from a given inoculum.

4. Addition of azide is necessary to inhibit the growth of aerobic oral bacteria and small gram-negative cocci. Addition of glucose, cystine, and sodium glutamate enhances growth. Tryptophane is also stimulatory but it was omitted from the above formula on account of its cost.

5. The degree of selectivity of this medium is fairly satisfactory for estimating the relative number of fusobacteria in the mouth. It is very doubtful that the Leptotrichia of Bøe will develop in this medium.

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AMINO ACID COMPOSITION OF PROTEINS OF HUMAN DENTIN

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IN VIEW of the evidence for bacterial hydrolysis and destruction of the organic matrix as a basic etiological factor in caries formation,[1, 2, 3] it is logical that the proteins of dentin and enamel should be examined as to their amino acid content, the sequence of their amino acids, and the mode of combination of the proteins with the chondroitin sulfate of the matrix. In this report the initial studies on the amino acid composition of the insoluble material of decalcified dentin are detailed. This particular aspect has been the subject of several investigations[4-7]; most of these investigations have employed technics that have revealed useful information but lack the accuracy and precision of the methods of Moore and Stein[8] as used in this study.

MATERIALS AND METHODS

Unerupted third molars and caries-free anterior teeth were placed over a desiccant immediately after extraction. Within 48 hours the teeth were thoroughly cleaned and were frozen. Preparation for separation of dentin was accomplished by breaking the teeth into large fragments, removing as much pulpal material as possible with cotton pliers, and grinding the large particles in a steel mill until the ground material would pass through an 80 mesh screen. After grinding, the powder was passed through a magnetic field to remove any metallic debris and the dentin was separated by the gravitational flotation method; the dentin sample was checked for purity by microscopic particle count.[9]

The dentin was demineralized by dialysis in a 5 per cent solution of EDTA (ethylenediaminetetraacetate) at pH 6.5. Completion of demineralization was verified by titration of samples of the EDTA solution with a standard magnesium chloride solution until a constant EDTA level was established. After demineralization was completed the residual dentin was washed with 50 per cent ethanol, absolute ethanol, anhydrous ether, and then dried in vacuo with phosphorus pentoxide at 100° C. until a constant weight was achieved; the ash content was found to be less than 0.1 per cent.

Several samples of the insoluble protein were used for the determination of ash and total nitrogen. Other samples of the protein were hydrolyzed in

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sealed tubes with 6 N hydrochloric acid at 100° C. for 24 hours. The hydrolysate was separated into its component amino acids with the 150 by 0.9 cm. Dowex 50-X4 column[8] and analyzed by the modified photometric ninhydrin method of Moore and Stein.[10] The identification of each peak was confirmed by paper chromatography.

RESULTS

The results of the analyses for amino acids (the average of three runs) are summarized in Fig. 1 and Table I. Our results are expressed as percentage of amino acid residue (a correction for the water of hydrolysis) and a 10 to 15 per cent increase in each value would need to be made for comparison with results of Hess and co-workers.[7] The results of Battistone and Burnett[4] (labeled, B & B in Table I) are also given for comparison as is a compilation of range of results for collagens from a variety of sources. Recent isolated results would extend the range for hydroxylysine down to about 0.5 per cent in some collagens.

TABLE I
AMINO ACID COMPOSITION OF DENTIN RESIDUE

COMPONENT	PRESENT STUDY (RESIDUE PER CENT)	HESS (PER CENT)	B & B (PER CENT)	COLLAGENS[10] (PER CENT)
Nitrogen	17.9	--	--	17.8 -18.4
Peptide A	2.85[1]	--	--	- -
Peptide B	3.44[2]	--	--	- -
Urea	0.39	--	--	- -
Hydroxyproline	15.69	13.10	14.00	9.1 -14.1
Aspartic acid	2.78 (5.63)[3]	5.63	6.60	6.7 - 7.1
Threonine	1.51	3.10	2.10	2.3 - 3.5
Serine	3.60	3.50	2.80	2.8 - 5.9
Proline	12.96	16.25	10.00	13.3 -16.5
Glutamic acid	5.82 (9.26)[4]	10.62	10.50	10.8 -12.2
Glycine	20.17	22.50	19.10	23.3 -29.6
Alanine	5.99	8.50	8.50	8.6 -10.0
Valine	2.28	3.00	2.60	2.3 - 3.2
Cystine	--	0.10	0.10	0.08- 0.12
Methionine	0.67	0.50	0.64	0.68- 1.16
Isoleucine	1.11	1.25	--	1.5 - 2.2
Leucine	2.74	3.60	--	3.0 - 3.7
Tyrosine	0.47	1.10	1.00	0.86- 1.06
Phenylalanine	2.06	1.90	--	2.3 - 2.6
Ammonia	0.64	--	--	- -
Hydroxylysine	2.71	1.00	1.10	1.1 - 1.7
Ornithine	0.86	--	--	- -
Lysine	2.58	3.34	3.00	4.9 - 5.7
Histidine	0.71	0.80	0.63	0.62- 0.94
Tryptophan	—[5]	--	--	- -
Arginine	6.66 (7.52)[6]	7.40	8.30	8.6 - 9.4
	100.07[7]	106.20[8]	99.17[9]	

(1) Contains aspartic acid; calculated as aspartic acid.
(2) Contains glutamic acid; calculated as glutamic acid.
(3) Total of aspartic acid and aspartic acid peptide.
(4) Total of glutamic acid and glutamic acid peptide.
(5) Tryptophan was present in our preparations at low levels; the values in an acid hydrolysate would be meaningless.
(6) A corrected value including ornithine.
(7) Total of residues; the total should be 100 per cent, i.e., correction was made for the water of hydrolysis of peptide bonds. The precision was ± 1 per cent.
(8) Not corrected for water of hydrolysis and expressed as grams amino acid per 100 grams protein.
(9) Contains 8.20 per cent as phenylalanine, leucine, and isoleucine.
(10) Range of value reported for collagens from a variety of sources; expressed as grams amino acid per 100 grams protein.

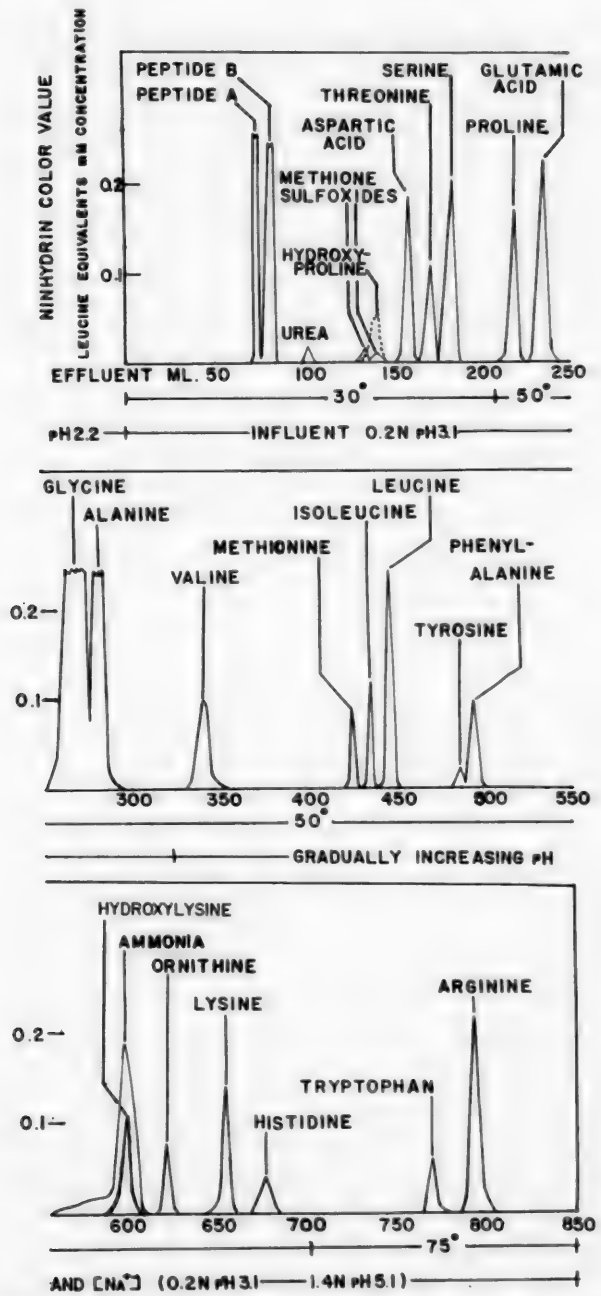

Fig. 1.—Amino acid analyses of dentinal protein; ninhydrin color values in millimols are plotted against milliliters of effluent.

Two peaks eluted from the column prior to the known amino acids were identified as peptides containing aspartic acid and glutamic acid; samples of the eluates from several columns were concentrated, deionized, and subjected to hydrolysis for 48 hr. at 100° C. with 6 N hydrochloric acid. Aspartic acid and glutamic acid, respectively, were identified as major components in the hydrolysates by paper chromatography (two dimensional, propanol-water then butanol-water). As sufficient material is accumulated, the hydrolysates will be subjected to quantitative column chromatography and further studies of the composition of the peptides will be made.

The differences between our analyses and those of others may be a reflection of the methods used. The laborious but accurate system of chromatography on crosslinked polystyrene resins is the accepted standard for such studies. However, the precision of plus or minus of one per cent in the total value should not be interpreted as the accuracy of the method. Quite probably, the accuracy for any one component does not exceed ± 5 per cent, even though recovery of added amino acids is possible at a better level of precision. Nevertheless, allowance for an error of 5 per cent would not change in any way the marked differences of dentinal collagen from other collagens (the higher content of the hydroxyaliphatic amino acids, hydroxylysine and hydroxyproline, and the lower content of the basic amino acids, arginine, and lysine). The analyses from other laboratories by different technics bear out most of these differences.

DISCUSSION

The analyses of the dentin residue have confirmed the conclusions of others that the material is closely related to the collagens. However, the collagens of human dentin differ from those from other sources in that there is a higher content of the hydroxyaliphatic amino acids, hydroxylysine, and hydroxyproline than in collagens from a variety of other sources. Several other amino acids, notably the basic amino acids, lysine and arginine, are found in lower concentration than in other collagens. A comparison of collagen from human dentin with that from human bone would seem to be indicated to rule out species differences but Hamilton and Anderson[11] have reported a content of 0.8 per cent for hydroxylysine in collagen from human bone.

The discovery of the peptide fractions resistant to hydrolysis (6 N hydrochloric acid for 24 hr. at 100° C.) is a unique finding of possible importance in an understanding of the composition of the organic matrix of dentin. The concentrations, as reported here, were quite reproducible for these hydrolysates. It is hoped that a delineation of the nature of the peptides will give a clue as to the combination of the collagen and the chondroitin sulfate components of the matrix.

SUMMARY

The insoluble protein of dentin was found to be a collagen characterized by a high content of glycine, proline, and hydroxyproline; the presence of hydroxylysine and hydroxyproline confirms the classification as a collagen. The dis-

covery of glutamic and aspartic acids in peptides in the acid hydrolysates is novel and may be a clue as to the combination of the collagens in the organic matrix of dentin. The collagen fraction of dentin was found to contain more of the hydroxyaliphatic amino acids, hydroxyproline, and hydroxylysine, and less of the basic amino acids, arginine and lysine, than collagens from other sources.

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THE RESPIRATION OF THE SALIVARY MICROBIAL FLORA AND ITS RELATION TO DENTAL CARIES ACTIVITY

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ALTHOUGH manometric methods for estimating the exchange of gases have been in use for generations,[1] it is only recently that they have been adapted to studies of the metabolic reactions of the oral microbial flora. Most such investigations have been concerned with determining if there is a significant difference in the endogenous respiration rates of caries-active as compared to caries-inactive saliva. Unfortunately, in the majority of these studies, there were an inadequate number of test subjects used; and an inadequate evaluation was made of their dental caries activity. Under these circumstances it is not surprising that there was considerable disagreement in the findings reported by various workers.

Hartles and McDonald,[2] Burnett[3, 4] and Neuwirth and Summerson[5] were unable to find any correlation between the rate of endogenous respiration and dental caries activity.

In contrast, Bramstedt, Vonderlinn, Naujoks, Kroncke, and Eggers-Lura[6-10] all reported that caries resistance was accompanied by a high endogenous respiration rate. Kroncke and Naujoks[11] claimed clinical success in using this difference as a new test for dental caries activity.

Calandra and Adams,[12] using the direct method of Warburg, tested the action of pooled whole saliva on several different substrates. They observed a rapid oxidation of lactate and slight oxidations of pyruvate, acetate, and propionate. They felt that the observed rapid oxidation of lactate might possibly account for those individuals who seem to have a natural immunity to dental caries. It was reasoned that the lactic acid formed by such persons might be rapidly oxidized to less harmful substances.

In view of the conflicting evidence reported by previous investigators and in order to examine further the hypothesis of Calandra and Adams, the present study was undertaken with the following objectives: (1) to determine if there is a positive correlation between dental caries activity and the endogenous respiration rate of the oral microbial flora; and (2) to determine if there is a correlation between dental caries activity and the rate of respiration of glucose, lactate, acetate, or pyruvate by the oral microbial flora.

The data in this paper are, in part, from a thesis submitted by the senior author to the Faculty of the Graduate School, Northwestern University, in partial fulfillment of the requirements for the degree of Master of Science (Bacteriology).

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MATERIALS AND METHODS

In order to assure the validity of the data, saliva was obtained from only those individuals whose examinations showed that they belonged to one of two extreme groups: either those who were extremely caries-active or those who were extremely caries-inactive. Past dental caries experience was determined by both clinical and radiographic examinations. Dental caries activity was measured by means of lactobacilli counts made on Rogosa's agar[13] and by Snyder's colorimetric test.[14] In this manner, 50 individuals were selected and divided into two groups of 25 each, as shown in Tables I and II.

Group I. Caries-Inactive.—In the case of each individual the Snyder's test indicated no caries activity; the lactobacillus count was zero; and there had been no history of dental caries experience for the past 2 years immediately prior to the study.

Group II. Caries-Active.—In the case of each individual the Snyder's test indicated marked caries activity; the lactobacillus count was 50,000 or more; and there was a history of at least 10 or more new carious lesions during the year immediately prior to the study.

Caries activity tests were conducted on duplicate salivary samples from each individual both prior to and at the time the respiration studies were performed. The endogenous respiration and the respiration of the various substrates by the microbial flora of each saliva sample were measured at 37.5° C. by the "direct method" of Warburg.[15, 16]

On the day upon which the respiration experiments were to be performed, 10 ml. of paraffin-stimulated saliva was collected before breakfast from each of 2 test individuals. Five separate 1.0 ml. aliquots were removed from each sample and placed in the main compartments of Warburg flasks along with 1.5 ml. of phosphate buffer (0.05 M, pH 7.2). The side arms of each set of five flasks contained the following: Flask 1—0.5 ml. phosphate buffer (0.05 M, pH 7.2); Flask 2—0.5 ml. of 0.1 M glucose*; Flask 3—0.5 ml. of 0.1 M sodium acetate; Flask 4—0.5 ml. of 0.1 M sodium pyruvate; and Flask 5—0.5 ml. of 0.1 M lithium lactate. Also, 0.2 ml. of a 19.7 per cent potassium hydroxide and a piece of fluted filter paper were placed in the center well of each flask.

The flasks were then attached to manometers and placed in the water bath of a Warburg respirometer. Following equilibration, the substrates from the side arms were introduced into the main vessels and readings commenced. Readings were recorded every 10 minutes for the first 60 minutes and every 20 minutes thereafter until the reactions were stopped.

RESULTS

The mean values for microliters of oxygen uptake were determined for the caries-active and the caries-inactive groups for each 20-minute interval following the addition of the substrates. The slopes of the straight lines that best

*All substrates were made up in phosphate buffer solutions (0.05 M, pH 7.2).

TABLE I
TEST SUBJECTS (GROUP I—CARIES INACTIVE)

NO.	SUBJECT	SEX	AGE	RACE	SNYDER TEST		MEAN LACTOBACILLI COUNT PER C.C.
					ACID	GAS	
1	T. S.	M	23	W	0	–	0
2	F. I.	M	24	W	0	–	0
3	C. K.	F	28	W	0	–	0
4	F. K.	M	31	W	0	–	0
5	G. G.	M	33	W	0	–	0
6	J. G.	F	36	W	0	–	0
7	G. E.	M	30	W	0	–	0
8	M. M.	F	28	W	0	–	0
9	A. M.	M	32	W	0	–	0
10	B. K.	F	27	W	0	–	0
11	J. M.	M	18	W	0	–	0
12	C. S.	F	31	W	0	–	0
13	J. H.	M	18	W	0	–	0
14	K. J.	M	17	W	0	–	0
15	J. R.	M	18	W	0	–	0
16	W. A.	M	19	W	0	–	0
17	B. B.	M	19	W	0	–	0
18	R. C.	M	17	W	0	–	0
19	I. S.	M	33	W	0	–	0
20	H. E.	M	33	W	0	–	0
21	L. O.	M	17	W	0	–	0
22	R. B.	M	17	W	0	–	0
23	I. E.	M	17	W	0	–	0
24	C. B.	M	17	W	0	–	0
25	E. M.	M	19	W	0	–	0

TABLE II
TEST SUBJECTS (GROUP II—CARIES ACTIVE)

NO.	SUBJECT	SEX	AGE	RACE	SNYDER TEST		MEAN LACTOBACILLI COUNT PER C.C.
					ACID	GAS	
26	R. S.	M	18	W	4+	+	852,000
27	J. L.	M	18	W	4+	+	98,000
28	A. C.	M	18	W	4+	+	322,000
29	R. M.	M	17	W	4+	+	160,000
30	B. J.	M	18	W	4+	+	189,000
31	M. R.	M	18	W	4+	+	210,000
32	E. W.	M	19	W	4+		128,000
33	R. R.	M	17	W	4+		79,000
34	C. W.	M	18	W	4+		51,000
35	J. P.	M	18	W	4+		190,000
36	J. C.	M	18	W	4+		145,000
37	F. M.	M	17	W	4+		83,000
38	J. F.	M	19	C	4+	+	91,000
39	D. V.	M	18	W	4+	+	278,000
40	W. W.	M	19	W	4+		104,000
41	E. G.	M	19	W	4+	+	742,000
42	R. H.	M	18	W	4+	+	86,000
43	H. W.	M	18	W	4+		120,000
44	J. M.	M	18	W	4+		96,000
45	W. K.	M	18	W	4+		112,000
46	J. B.	M	18	W	4+	+	162,000
47	G. B.	M	19	W	4+	+	145,000
48	F. Mc.	M	19	W	4+		93,000
49	J. R.	M	18	W	4+	+	63,000
50	L. M.	M	18	W	4+	+	120,000

fitted these mean Q_{O_2} values for the endogenous and substrate respiration were calculated according to the method of least squares[17] and graphs constructed. The "t" test was applied to determine the significance of these differences. The "t" value is the ratio of the difference to its standard error or the Significance Ratio. A table[17] (called the t table) has been devised, which gives for various degrees of freedom (n) the probability (P) of significance. For these comparisons where n = 8, a "t" value greater than 5.041 is highly significant; the

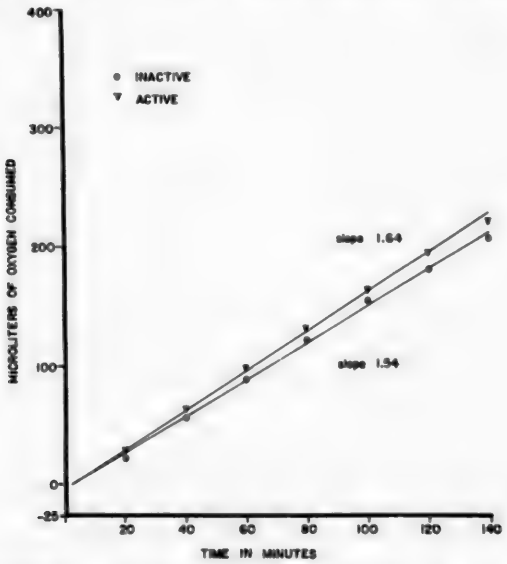

Fig. 1.—A comparison of the mean endogenous respiration rate of the caries-active group with that of the caries-inactive group.

probability being less than one time in 1,000 that such a difference could be due to chance.

Fig. 1 shows the slopes obtained for the endogenous respiration of the two groups. In spite of the fact that the 50 individuals used were selected to represent the extremes of caries activity and inactivity, the statistical analysis of these values (Table III) failed to reveal any significant difference between the two groups. These findings are in sharp contrast to those[6-11] which revealed a difference great enough to be used as a new test for dental caries activity.

The slopes of the mean respiration rates of the caries-inactive group for the four substrates tested are shown in Fig. 2. It can be seen that there was no oxidation of acetate and only a very slight oxidation of pyruvate (t = 9.30). It is interesting to note (Table IV) the considerable difference between the rates of respiration of glucose (t = 47.34) and lactate (t = 15.05). The much slower oxidation of lactate by this group would tend to rule out the hypothesis of Calandra and Adams that a rapid oxidation of this substrate might be responsible for the caries inactivity of these individuals. The slopes obtained for the

TABLE III
COMPARISON OF RESPIRATORY ACTIVITY BETWEEN THE TWO GROUPS FOR THE FIRST 60 MINUTES

SUBSTRATE	GROUP I INACTIVE b_1	GROUP II ACTIVE b_2	AVERAGE STANDARD DEVIATION $\bar{S}_{y.x}$	STANDARD ERROR $S_{b_2-b_1}$	t	P
Endogenous respiration	1.59	1.69	2.795	± .0934	1.07	$P > 0.3$
Acetate	1.57	1.64	2.140	± .0514	0.96	$P > 0.3$
Pyruvate	0.067*	0.44*	0.400	± .0095	27.95	$P < 0.001$
Lactate	0.46*	2.09*	1.145	± .0387	42.14	$P < 0.001$
Glucose	0.98*	2.44*	0.832	± .0458	31.87	$P < 0.001$

*Corrected for endogenous respiration.

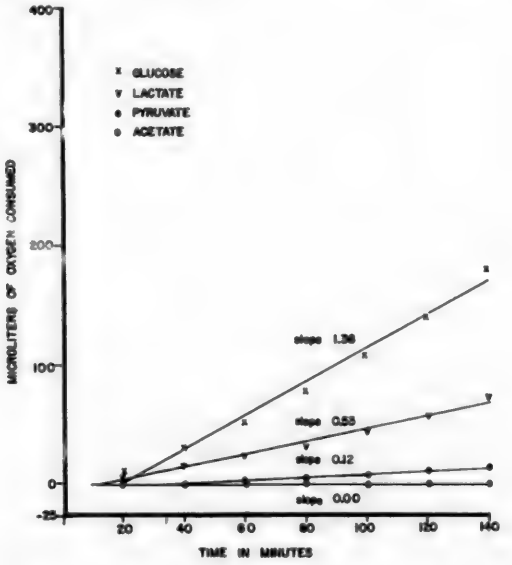

Fig. 2.—The mean respiration rates of the caries-inactive group for the four substrates tested.

mean respiration rates of the caries-active group for the four substrates appear in Fig. 3. As in the case of the caries-inactive group, there was no oxidation of acetate. There was a moderate respiration of pyruvate ($t = 38.86$), while the respiration of both glucose ($t = 128.42$) and lactate ($t = 87.82$) was extremely rapid. A comparison (Table III) of the difference between the slopes of the mean Q_{O_2} values of the caries-inactive group (b_1) with those of

TABLE IV
COMPARISON OF "t" VALUES WITHIN EACH GROUP FOR THE FIRST 60 MINUTES

SUBSTRATES	GROUP I INACTIVE	P	GROUP II ACTIVE	P
Endogenous vs. Pyruvate	9.30	$P < 0.001$	38.86	$P < 0.001$
Endogenous vs. Lactate	15.05	$P < 0.001$	87.82	$P < 0.001$
Endogenous vs. Glucose	47.34	$P < 0.001$	128.42	$P < 0.001$
Pyruvate vs. Lactate	4.05	$P < 0.01$	80.50	$P < 0.001$
Lactate vs. Glucose	14.16	$P < 0.001$	16.28	$P < 0.001$

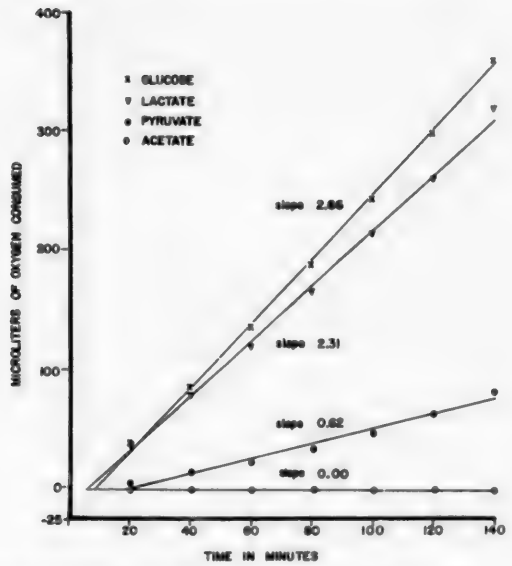

Fig. 3.—The mean respiration rates of the caries-active group for the four substrates tested.

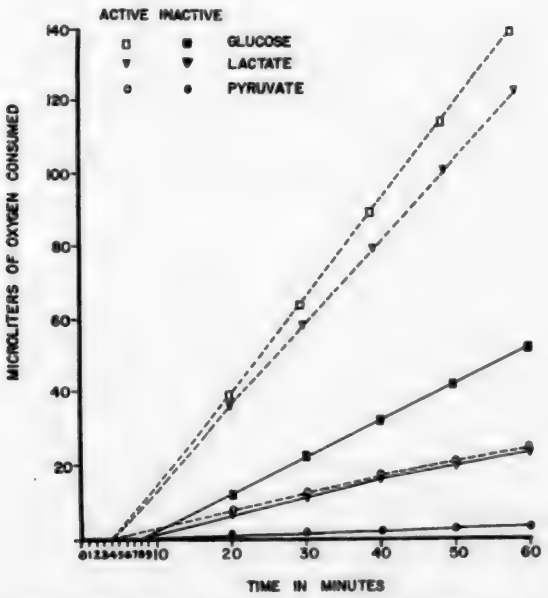

Fig. 4.—A comparison of the time of initiation and rate of oxidation of glucose, lactate, and pyruvate by the caries-active and the caries-inactive groups.

the caries-active group (b_a) shows that the respiration rates of the caries-active group were significantly higher for all three of these substrates.

An enlarged view, in which the Q_{O_2} values of both groups may be compared, is shown in Fig. 4. In addition, this figure shows that there was a 4-minute difference found in the time at which the two groups commenced to oxidize these substrates. The caries-inactive group did not commence to oxidize these substrates until 4 minutes after such oxidation had been initiated by the caries-active group.

DISCUSSION

The rapid oxidation of glucose by caries-active saliva and the considerably slower reaction of caries-inactive saliva on this substrate could logically have been expected, although it was not previously found by others.[3, 4, 5, 9, 10] Neuwirth and Summerson and many others[5, 18, 19] have shown that acid, especially lactic acid, is a major product of this oxidation. However, intensive investigations into the salivary pH and its relation to dental caries activity by numerous workers[20-23] and others have failed to demonstrate any correlation between salivary pH and dental caries activity. This is most probably due to the fact, as demonstrated in this study, that lactate is so rapidly oxidized by caries-active saliva.

The question that remains to be answered is the persistence of dental caries activity in the face of the rapid oxidation of lactate exhibited by caries-active saliva. It has been demonstrated by numerous investigators that there is a rapid decrease in the pH of the dental plaques of caries-active individuals following a sugar mouth-rinse[24-28] and that this is due mainly to its lactic acid content.[29, 30] It has been shown by others[31, 32, 33] that lactic acid, as well as glucose, can penetrate the dental plaque and there be protected from the saliva. The explanation may then be in this ability of the lactic acid formed to penetrate the dental plaque and there be protected from further oxidation by the aerobic microbial flora.

An equally important finding in this study was the fact that caries-active saliva commenced to oxidize the substrates as soon as they were added, whereas the caries-inactive saliva did not initiate such oxidation until 4 minutes later. This difference could become of importance followng the ingestion of sweets and might account for the fact that the pH is not decreased, to any appreciable extent, in the dental plaques of caries-inactive individuals following a sugar mouth-rinse.[26, 27, 33, 34]

SUMMARY

1. No correlation was found between the endogenous respiration rates of the salivary microbial flora and dental caries activity.

2. Acetate was not oxidized by the salivary microbial flora, but a positive correlation was found between dental caries activity and the rate of oxidation of glucose, lactate, and pyruvate. Caries-active saliva exhibited an extremely rapid oxidation of these substrates as compared to that of caries-inactive saliva.

3. A positive correlation was demonstrated between the state of dental caries activity and the time at which the oxidation of the substrates was commenced.

4. It is believed that a combination of these findings could be used as a basis for development of a new and accurate test for dental caries activity.

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HEREDITY AND DENTAL CARIES

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INTRODUCTION

THE literature provides considerable evidence to demonstrate the influence of diet upon the incidence of dental caries, but it is also clear from many studies that individual variation in susceptibility to caries exists, even under identical controlled conditions. For example, Gustafsson, Quensel, Lanke, Lundqvist, Grahnen, Bonow, and Krasse[1] observed that some individuals, consuming a diet known to be caries producing, showed no increase in carious lesions, while others living on a virtually sugar-free, low carbohydrate diet developed as many as nine new lesions per year. They suggested that types of caries may exist, in which sugar and other refined carbohydrates play a relatively minor causative role. Among the factors to be considered in seeking an explanation of this, the possibility that a constitutional factor may be involved cannot be ignored.

Familial similarity in dental caries experience has been shown by Klein and Palmer,[2] and Klein[3, 4] concluded, from a study of dental caries in parents and children, that it was difficult to exclude the view that susceptibility to dental caries in children involves strong familial factors which may have a genetic basis, possibly sex-linked.

However, in animals, more precise direct evidence has been obtained[5] to show that, in the laboratory rat, heredity is a factor in determining resistance or susceptibility to dental caries under uniform environmental conditions. By selective breeding, Hunt and his co-workers produced two distinct strains of albino rats, one caries-susceptible and the other caries-resistant.

The evidence that susceptibility and resistance to dental caries in rats can be inherited clearly illustrates the existence of such a biologic mechanism and thus suggests the possibility that genetic constitution may also be a factor influencing susceptibility and resistance to caries in man.

For the further elucidation of this problem, twins provide valuable material. Bachrach and Young[6] made a study of dental caries in monozygous (''identical'') and dizygous (''fraternal'') twins in London schools and, although the similarity in caries between twins was found to be greater in monozygous than

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in dizygous pairs, the difference between the two types of twins was not regarded as statistically significant. These findings were interpreted to suggest that heredity played a minor part in the etiology of dental caries.

Goldberg,[7] from a study of 42 pairs of monozygous twins, concluded that heredity influences dental caries indirectly by influencing tooth morphology, especially the formation of pit and fissure systems.

Dahlberg and Dahlberg[8] investigated dental caries experienced in twins and concluded that inherited factors are at the least half as important and, at the most, of equal importance as environmental factors in the incidence of this disease.

The differing conclusions reached by Bachrach and Young and Dahlberg and Dahlberg provided the main reason for this study. Furthermore, the environment of twins is inevitably very similar, especially in childhood, and since environmental factors (particularly diet) are known to exert an important influence on the incidence of dental caries, it was thought that a comparison of the differences in caries experience which occur between *unrelated* pairs of children and the differences which occur between twins would provide a better estimate of the contribution of environment and hence permit a better evaluation of any possible genetic effects.

For these reasons, therefore, the present study was undertaken to investigate the dental-caries experience of identical and fraternal twins and, by comparison, to attempt to assess the relative importance of heredity and environment as factors in the causation of this disease. In this way, it was hoped that it might be possible to distinguish genetic, familial, and purely environmental influences in the production of dental caries, and to obtain some indication of their magnitude and possible significance.

From birth, the total environment of monozygous pairs can be assumed in general to differ neither more nor less than does the environment of dizygous pairs. In this study, therefore, it is assumed that postnatal environmental differences between members of twin pairs are approximately equal, whether the pairs are monozygous or dizygous.

METHODS

With the cooperation of the Chief Executive School Medical Officer and the Chief Dental Officer for Edinburgh, the head teachers of all schools under the Local Authority were asked to submit a list of all twins on their school register. Like-sex twins only were selected from these lists, and from a total of 232 like-sex pairs of twins, listed by the head teachers, 224 pairs were examined.

The children were examined in the schools during school hours and the examinations were made using plane mouth mirrors and Ash No. 54 probes. Illumination was provided by a portable lamp using a 60 watt bulb. Carious lesions and fillings were recorded for each tooth and for each tooth surface involved. All missing teeth were recorded, together with any relevant dental history which could be obtained, and all unerupted teeth were recorded as such.

Diagnosis of Type of Twin.—

Similarity method: The criteria for classifying monozygous twins as defined by Newman, Freeman and Holzinger[9] were closely followed in making the diagnosis of twin type in this study, including a comparison of fingerprints. Therefore, for each pair of twins examined, nine physical features were compared together with an assessment of general appearance. The characters compared were nose, lip and chin shape; hair color and texture; eye color (including consideration of iris pattern); hands, shape and proportions, including nails; ears; and tooth color and shape. For those features which are bilateral, i.e., hands and ears, a double comparison was always made. The right of one twin was compared with the right of the other twin, and the left of one with the left of the other; then the opposite sides were compared, i.e., right of one with left of other and vice versa. This was done as a routine in twins to make allowance for reversal of asymmetries. These characters, together with "General Appearance," made up twelve categories and the findings in respect of these were recorded under the headings "Identical," "Similar," and "No Similarity." To obtain a numerical assessment of these, a value of 2 was allotted to each feature under the headings "Identical," and a value of one to each feature recorded under the heading "Similar." Hands and ears, as bilateral characters, contributed four categories in all. No value was allotted to any feature recorded under "No Similarity."

Since "Identical," "Similar," and "No Similarity" are in respect of any one feature mutually exclusive, the total score possible for complete identity of the characters studied was 24.

Interpretation of fingerprints: Each print was examined with a hand-magnifying glass and compared with the corresponding print for the other twin. The criteria of similarity, used by Stocks,[10] were adopted and prints were classed as similar not only in respect to type, e.g., arch, whorl or loop, but also in regard to the general pattern, inclination of axes, position of deltas, and apparent numbers of ridges which made them appear the same on examination.

Newman and his associates[9] concluded that the observation of seven or more corresponding fingerprints in a pair of twins would make the diagnosis of monozygous twins certain, and that standard was adopted in this study.

Examination of fingerprints revealed reversal (that is, the right hand of one twin resembling the left hand of the other) in 13 pairs of monozygous and none in dizygous twins. A careful examination of the dental records of these children failed to reveal any clear evidence of reversal in pattern of dental caries. Therefore, in the tooth-by-tooth comparisons which follow, the question of possible reversal of comparable teeth was ignored. Thus, by these methods, the twins were classified as monozygous ("Identical") and dizygous ("Fraternal"). The findings for physical resemblance and fingerprint correspondence for both types of twins are given in Table I as mean values.

TABLE I
MEAN PHYSICAL RESEMBLANCE AND FINGERPRINT CORRESPONDENCE IN MONOZYGOUS AND DIZYGOUS TWINS

	PHYSICAL RESEMBLANCE (MAXIMUM VALUE FOR IDENTICAL RESEMBLANCE = 24)	FINGERPRINT CORRESPONDENCE (MAXIMUM FOR COMPLETE CORRESPONDENCE = 10)	
		CROSS RESEMBLANCE*	INTERNAL RESEMBLANCE*
Monozygous	21.7	7.4	4.7
Dizygous	5.6	2.1	5.5

*Cross resemblance in fingerprints is the resemblance between corresponding fingers of each twin, while internal resemblance is the resemblance between right and left hands of the twin.

The distribution of twins by age and type of twin is shown in Table II.

TABLE II
DISTRIBUTION OF LIKE-SEX TWINS BY AGE AND TYPE OF TWIN

AGE	MONOZYGOUS	DIZYGOUS
5	7	8
6	17	22
7	15	19
8	9	11
9	8	8
10	12	18
11	12	22
12	2	2
13	4	8
14	7	6
15	2	2
16	1	1
17		1
Totals	96	128

The twin pairs having been separated into their two groups, the individual twins of a pair were designated "Twin A" and "Twin B." Where any disparity existed in the number of permanent teeth erupted, the twin who had the lesser number of teeth was designated the "A" (or registration) twin, otherwise the designations A and B were made according to the alphabetical order of christian names.

Control Groups.—The object of the study was to compare similarity of dental caries experience in monozygous and dizygous twins, and also to compare the experience of unrelated pairs of children with each type of twin in respect to this disease. Therefore, a control group was created by selecting the dental record of a child of the same age, sex, and number of teeth erupted as the "A" (registration) twin. Thus, there was one control child for each pair of twins (see Table III). These "control" children were selected from a sample of over 4,000 Edinburgh children examined during the previous 2 years.

Each "control" child so selected was then paired with the "A" twin to create a pair of children in no way related but as alike as possible in respect to age and sex, and with a difference in the number of permanent teeth erupted no greater than existed between twins A and B. The variable *not* held constant

was their dental-caries experience. The two control groups have been designated the "monozygous control" and the "dizygous control" groups.

Four groups were thus created, the monozygous twins, the dizygous twins, and two groups of unrelated pairs.

The mean age of the twins and their control groups are given in Table III and show that differences in age between the twins and their controls are negligible.

TABLE III
MEAN AGE OF TWIN AND CONTROL GROUPS

GROUP	NUMBER	MEAN AGE (YEARS)	STANDARD DEVIATION OF MEAN (YEARS)
Monozygous	96 (pairs)	9.47	2.88
Monozygous control	96	9.50	2.86
Dizygous	128 (pairs)	9.50	3.00
Dizygous control	128	9.55	2.74

With regard to caries prevalence, no significant differences were found between the twins and their controls, and the relevant data are summarized in Table IV.

TABLE IV
MEAN DECAYED, MISSING AND FILLED TEETH IN TWINS AND CONTROLS

	PERMANENT TEETH		DECIDUOUS CANINE AND MOLAR TEETH	
	NO. OF CHILDREN	MEAN DMF PER CHILD	NO. OF CHILDREN*	MEAN DMF PER CHILD
Monozygous	176†	2.79	90	5.39
Monozygous control	88	2.74	45	5.50
Dizygous	242†	2.59	116	5.51
Dizygous control	121	2.66	58	5.76

*DMF values for deciduous teeth were calculated only for those under 8.99 years of age.
†Out of the total number of twins, 8 monozygous pairs and 7 dizygous pairs had no permanent teeth erupted.

The data were now arranged so that for each twin pair A and B and for the selected control child (designated C) the state of each corresponding tooth could be compared simultaneously. For example, the lower right permanent first molar of twin B, and also that of A were compared with that of C.

The state of each tooth was recorded as "carious" or "noncarious," using the same criteria as for the DMF index.

RESULTS

From these data were computed the number of occasions on which corresponding teeth in the twins and their controls were in "like" or "unlike" carious state. By this method of analysis, there arose four possible combinations of dental caries experience between the twins and their controls (Table V).

TABLE V

	TWINS PAIR		CONTROL
	A	B	C
		(Caries + or −)	
(i) Concordance in twins (AB) with discordance in controls (AC)	+	+	−
	−	−	+
(ii) Discordance in twins (AB) with concordance in controls (AC)	−	+	−
	+	−	+
(iii) Concordance in twins (AB) with concordance in controls (AC)	+	+	+
	−	−	−
(iv) Discordance in twins (AB) with discordance in controls (AC)	+	−	−
	−	+	+

The results of these comparisons are presented in Tables VI and VII.

From Table VI it can be seen that concordance in the monozygous twins and discordance between control pairs is greater than the expected value; accordingly, in the dizygous twins it is less than the expected value.

Conversely, discordance in monozygous twins with concurrent concordance in the control pairs is less than expected, while for the dizygous it is greater.

The high number of observations of concordance in both twins *and* controls concurrently are due largely to the recording of noncarious teeth as concordant, and to a lesser extent to caries of the corresponding teeth.

TABLE VI
CONCORDANCE AND DISCORDANCE IN DENTAL CARIES IN CORRESPONDING PERMANENT TEETH OF TWINS AND THEIR CONTROLS

		MONOZYGOUS TWINS AND CONTROLS	DIZYGOUS TWINS AND CONTROLS
(i) Concordance in twins with discordance in controls	Observed	155	183
	(Expected)	(148)	(190)
(ii) Discordance in twins with concordance in controls	Observed	62	97
	(Expected)	(70)	(89)
(iii) Concordance in twins with concordance in controls	Observed	1014	1281
	(Expected)	(1005)	(1290)
(iv) Discordance in twins with discordance in controls	Observed	64	100
	(Expected)	(72)	(92)

$\chi^2 = 3.73$ $(0.30>p>0.20)$.

In general, the data revealed a higher concordance in monozygous than in dizygous twins. The differences between the two types of twins are small and are not statistically significant.

The data presented in Table VII, relating to the deciduous teeth, follow the trends shown in Table VI with one exception, namely, that the observed concordance in twins with concurrent discordance in controls is less than the expected value.

Examination of the individual χ^2 values showed that for both permanent and deciduous teeth, the greatest contribution to the total χ^2 was made by the category "concordance in twins with concordance in controls" and that monozygous twins had higher observed values than expected

TABLE VII
CONCORDANCE AND DISCORDANCE IN DENTAL CARIES EXPERIENCES IN CORRESPONDING DECIDUOUS TEETH OF TWINS AND THEIR CONTROLS

		MONOZYGOUS TWINS AND CONTROLS	DIZYGOUS TWINS AND CONTROLS
(i) Concordance in twins with discordance in controls	Observed (Expected)	137 (139)	187 (185)
(ii) Discordance in twins with concordance in controls	Observed (Expected)	52 (62)	92 (82)
(iii) Concordance in twins with concordance in controls	Observed (Expected)	287 (274)	352 (365)
(iv) Discordance in twins with discordance in controls	Observed (Expected)	64 (66)	89 (87)

$\chi^2 = 3.90$ $(0.30>p>0.20)$.

By inspection, it can be seen from Table VI that for both types of twins, concordance between twins with discordance between control pairs is high (monozygous = 155, dizygous = 183), while discordance between twins with concordance between controls is low (monozygous = 62, dizygous = 97).

These differences were found to be highly significant for both permanent and deciduous teeth, and the probability that this could occur by chance was less than 1 in 1,000 in both instances.

Comparison With Previous Studies.—Bachrach and Young[6] presented data relating to the similarity of dental caries experience in twins. Much of their data are not, however, comparable with that derived from this present study, but for those data which can be compared a considerable measure of agreement is found.

From the total number of tooth pairs examined, it was possible to derive the numbers of pairs in which there was discordance between the twins (i.e., when only one of the tooth pairs was carious). Table VIII and Fig. 1 present these data for the Edinburgh twins and for those studied by Bachrach and Young.

The study made by Bachrach and Young[6] is of interest in comparison with the present study, for in spite of differences in evaluating dental caries experience, their results and those of this study show a considerable measure of agreement.

TABLE VIII
DISCORDANCE IN DENTAL CARIES IN CORRESPONDING PERMANENT FIRST MOLARS IN TWINS AND CONTROLS

	BACHRACH AND YOUNG		EDINBURGH STUDY	
GROUP	NUMBER OF TOOTH PAIRS	TOOTH PAIRS DISCORDANT (%)	NUMBER OF TOOTH PAIRS	TOOTH PAIRS DISCORDANT (%)
Monozygous	449	25.2 ± 2.0	333	24.3 ± 2.3
Dizygous (like-sex)	318	32.1 ± 2.6	448	33.3 ± 2.2
Monozygous control group	—	—	327	41.3 ± 2.7
Dizygous control group	—	—	435	45.5 ± 2.4

Age. The mean age of the twins studied by Bachrach and Young was stated to be 9 years; that of the Edinburgh twins was 9.5 years.

Dahlberg and Dahlberg[8] also showed that discordance in dental caries between corresponding teeth was less in monozygous than in like-sex dizygous twins. This is in agreement with the findings of Bachrach and Young, and of those for the Edinburgh study. The similarity between Dahlberg and Dahlberg's findings and those of this present investigation are of interest. The percentage for all permanent teeth in which only one of a pair of corresponding teeth in twins was carious is given below in Table IX for Dahlberg and Dahlberg's data and for the present study.

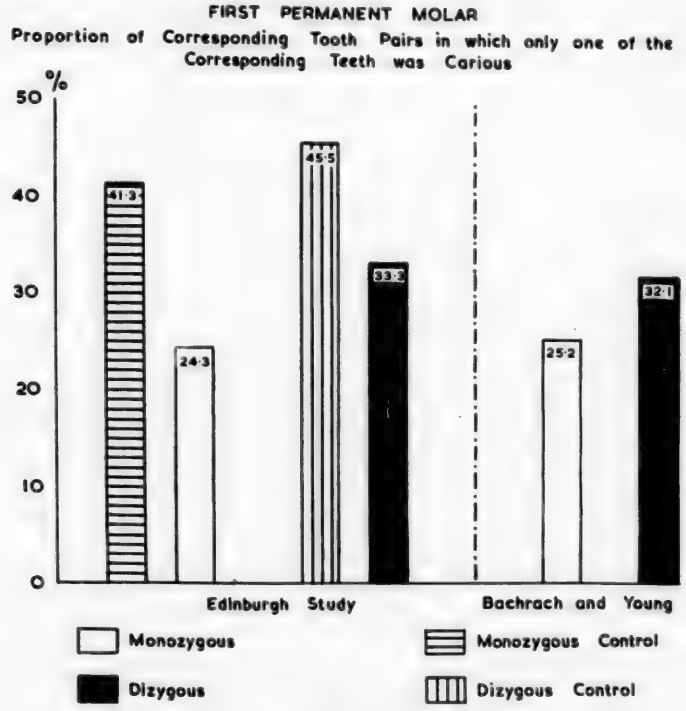

Fig. 1.

Although Dahlberg and Dahlberg found that the difference they observed between monozygous and like-sex dizygous twins was not statistically significant, in a further analysis of their data they calculated, by mathematical formulas,

TABLE IX
DISCORDANCE IN DENTAL CARIES IN CORRESPONDING PERMANENT TEETH IN TWINS

	DAHLBERG AND DAHLBERG		EDINBURGH STUDY	
GROUP	NUMBER OF TOOTH PAIRS	DISCORDANT TOOTH PAIRS (%)	NUMBER OF TOOTH PAIRS	DISCORDANT TOOTH PAIRS (%)
Monozygous	641	9.2 ± 1.1	1,295	9.7 ± 0.8
Dizygous (like-sex)	746	11.3 ± 1.1	1,672	12.5 ± 0.8

Ages. The mean age of the twins studied by Dahlberg and Dahlberg was, for the monozygous twins, 9.9 years and for the dizygous twins, 10.2 years.

comparative values of genetic and environmental factors and from these concluded that the ratio of genetic to environmental influences in the incidence of dental caries was at least 1:2 and that, therefore, the genetic factor appeared to be by no means negligible.

DISCUSSION

The present study was based upon the knowledge that monozygous twins have an identical hereditary constitution, while dizygous twins each possess their own distinctive genotype and are no more alike than ordinary siblings. Furthermore, it has been assumed in this study that the postnatal environment shared by the twins is equal, whether they are monozygous or dizygous.

Differences between monozygous twins and their control pairs may be due to

(a) the common environment of the twins, compared with the dissimilar environment of the control pairs,

and

(b) the identical hereditary constitutions of the twins compared with the dissimilar heredity of the controls,

while differences between dizygous twins and their control pairs can be regarded as due only to (a) the shared environment of the twins compared with the dissimilar environment of the control pairs.

This distinction being so, it could be expected that if constitutional factors do, in fact, exert any influence in determining susceptibility to dental caries, a comparison of the similarity of dental-caries experience in monozygous and dizygous twins should show monozygous twins to be more alike than dizygous.

However, the wealth of evidence presented in the literature relating to dental caries provides clear indication that environmental factors, especially diet, play a major part in the etiology of this disease and, therefore, if any genetic influence existed, it would seem likely to be small.

The results of this study show that between both types of twins and their respective controls the differences in similarity of caries experience were very highly significant, the twins showing high concordance and low discordance in dental caries, while the controls showed low concordance and high discordance. However, the data indicated that, while the monozygous twins differed from their control pairs to a greater extent than did the dizygous from their controls, the probability that this could be a chance occurrence was approximately 1 in 5 for both the permanent and deciduous dentitions.

The highly significant difference between all twins and the control pairs provides convincing evidence that the shared environment of the twins resulted in a greater similarity in their dental-caries experience, while the differing environment of the unrelated pairs resulted in a considerable difference in dental caries between the pair members.

From this evidence it can be concluded that environmental conditions constitute a major factor in the etiology of this disease.

The comparison of this study of twins with the two earlier studies shows a remarkable degree of agreement in those aspects which could be compared. In terms of numerical values the likeness is noteworthy and, furthermore, in all three studies the similarity in caries experience in monozygous twins was greater than that in dizygous twins.

In each of these three studies by themselves, the difference between the two types of twins in dental-caries experience was not of such a magnitude as to be statistically significant. Taken together, however, they provide consistent findings which seem to warrant the conclusion that the greater similarity in dental caries experience between monozygous as compared with dizygous twins is, in fact, real and not due to chance occurrence and, hence, that a genetic influence exists in the etiology of dental caries.

Nevertheless, the use of matched control pairs provided significant evidence to show that environment plays the major part in determining susceptibility to dental caries. This latter finding was derived from the differences observed between twins with their common environment and unrelatel pairs of children, each with his own individual environment.

The extent of the differences found between twins and the unrelated pairs of children was unexpected when viewed against the background of the relatively homogeneous environment of modern urban civilization. It suggests either that greater differences in individual environment exist than might be expected, or that susceptibility to dental caries is very sensitive to relatively minor environmental differences.

SUMMARY AND CONCLUSIONS

A study of dental caries in identical and like-sex fraternal twins was made in an attempt to assess the relative importance of heredity and environment as factors in the causation of this disease.

A total of 224 pairs of like-sex twins, in the age range of 5 to 17 years, was examined. They were classified as identical or fraternal pairs on the basis of physical resemblance and an analysis of fingerprints. Of the total number of twins, 96 pairs were classed as identical and 128 pairs as fraternal. For each pair of twins, an unrelated child was selected of the same age, sex, and possessing the same number of teeth erupted as one of the twins. This child, paired with one twin, thus provided a control pair. By comparing corresponding teeth of a pair, a comparison was made of the resemblance in dental-caries experience between identical twins with that which occurred between fraternal twins and also that between the unrelated pairs.

The data showed that resemblance in caries experience between identical twins is greater than between fraternal twins, while the unrelated pairs of children showed less resemblance than either type of twin.

Previous studies of dental caries in twins were discussed. The findings were compared with those from this study and a considerable measure of agreement found. By amplifying the earlier findings, the data from this present

study would seem to permit the conclusion that environmental factors clearly have greater influence, but that genetic factors also contribute to the causation of dental caries.

I should like to thank Professor J. H. F. Brotherston and Dr. Cecil Gordon for their interest and most helpful criticism. I am also indebted to Dr. Barnet Woolf for his statistical advice and to Mr. Geoffrey Moody, Chief Dental Officer for Edinburgh, for his valuable cooperation in the collection of the original data.

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A METHOD FOR DETERMINING THE EFFICIENCY OF LOCAL ANESTHESIA

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THE search for methods useful in the measurement of properties of drugs goes on continuously, and the end of the search for a procedure for the appraisal of the properties of local anesthetics has not been reached. A good objective method for evaluating local anesthetics was described by Bennett, Wagner, and McIntyre in 1942.[1] Their testing procedure of measuring directly from a nerve formed the starting point of this investigation. Such measurement eliminates many variables and offers an excellent criterion for observing the status of conduction.

PROCEDURE

Since such direct observations cannot be made readily from a human subject, a suitable animal was utilized. After preliminary standards were established, 27 cats were anesthetized by intraperitoneal injection of 50 mg. of pentobarbital sodium per kilogram of body weight. This dose was fortified by small repeated injections during the experiment, if necessary. The hair was clipped from the head and neck. Tracheotomy was performed and a metal tube connected to a breathing apparatus was inserted into the larynx to ensure a patent airway. The animal was then placed on a modified stereotaxic instrument. Two electrocardiographic electrodes were properly applied, and the heartbeat was audibly amplified throughout the experiment. Rectal temperature was recorded constantly. In this manner the progress of the animal was followed throughout the experiment.

A mid-line incision was made in the head and approximately 2 sq. cm. of bone was removed with a trephine and rongeurs. After dissection of the dura from the brain tissue, a left hemispherectomy was done to permit adequate exposure of the gasserian ganglion. Nichrome wire pick-up electrodes insulated with epoxylite and bared 1 mm. at the tip were lowered approximately 1 mm. into the left ganglion to record impulses conducted over the left maxillary division of the trigeminal nerve. The left side of the upper lip was sutured back and two silver stimulating needle electrodes were inserted supraperiosteally into the gingiva of the left maxillary canine fossa.

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Since the fifth cranial nerve is of major significance to the dentist, because it supplies the major sensory innervation to the oral cavity, it was decided to evaluate its behavior in this study. The maxillary division of this nerve was selected for this work because of its pure sensory characteristics and its accessibility. The mandibular division was not used because the presence of motor as well as sensory fibers in this nerve might complicate the analysis of anesthesia.

Stimulation to the terminal maxillary nerve fibers in the gingiva was provided by a model S-4c Grass stimulator. Current was directed to a stimulus isolation unit which next transmitted it to the stimulating electrodes in the gingiva. The response of the nerve was passed from the recording electrodes in the gasserian ganglion to six-channel Offner amplifiers. The amplifiers were in series with a type 279 Dumont dual-beam, cathode-ray oscilloscope and the action potential was observed and photographed with a Fairchild oscillograph camera for a permanent record.

Fig. 1.—The initial rise (*a*) from the base line is the shock or electrical artifact. This is independent of the true nerve response and is present regardless of nervous activity. It is always present and used as a point of reference when analyzing the data. Following this artifact is the true response (*b*). Following the injection of local anesthesia, this response disappears. The length of time that it takes to disappear can be measured. The length of time necessary for it to reappear can also be measured. The remaining electrical activity (*c*) is termed the base line and offers good evidence for stability of the experiment. *d* is a known selected time signal, in this instance 1,000 cycles per second, and this can be used for measuring nerve conduction.

The action potential represents the key measuring device in this study (Fig. 1). Activity which ensues when a nerve is stimulated mechanically, thermally, chemically, or electrically is composed of a chain of processes giving electrical potentials. The algebraic sum of these potentials is called the "action potential."[2] The action potential seen on the cathode-ray oscilloscope was used as the index of anesthesia in this work.

The stability of each animal preparation, apparatus, and action potential was observed and adjusted before the local anesthetic agent was administered. Three photographs were taken under standard conditions when the action potential had remained constant for 10 minutes or longer. An attempt was made to keep the delay, duration, and voltage readings of the current supplied by the stimulator as constant as possible.

The local anesthetic selected was then infiltrated approximately 1 cm. posterior to the stimulating electrodes with a 26-gauge needle connected by a plastic cannula to a tuberculin syringe. The rate of infiltration was kept constant at 15 seconds. The terminal fibers of the maxillary division of the trigeminal nerve were stimulated every 5 seconds during administration of the drug and at specified intervals during the recovery phase of the anesthesia.

A photographic record was made of the change in action potential showing the onset of anesthesia and the beginning of complete anesthesia, the initial return of nerve response, and the duration of anesthesia.

The animals were divided into three groups according to the volume of drug infiltrated. Two minims (0.12 ml.) were injected into Group 1, 4 minims (0.25 ml.) into Group 2, and 8 minims (0.49 ml.) into Group 3. These quantities were selected because preliminary experimentation had indicated that greater volumes caused abnormal expansion of the tissue and extensive spread of the solution.

One and two per cent concentrations of procaine hydrochloride and lidocaine hydrochloride were selected to establish the validity and reliability of the method. The local anesthetic solutions used did not contain any vasoconstrictors. The desire to eliminate as many variables as possible prompted this decision.

Ringer's solution and distilled water were utilized in the control phase of the experiment. The injection of 2, 4, and 8 minims of these solutions did not produce a change in the action potential. When excessive amounts of solution, 1 or 2 c.c., were injected, a temporary interference in nerve response occurred. This seemed to be due to the excessive amount of solution deposited in a small area of tissue. The solution would spread and produce a mechanical interference in the region of the stimulating electrodes. In the case of Ringer's solution, a chemical change of brief duration did occur.

This procedure was designed to study four parameters of local anesthesia: (1) onset of anesthesia, (2) onset of complete anesthesia, (3) initial return of nerve activity, and (4) final or complete return of sensory nerve activity.

RESULTS

The various stages and disposition of the action potential following injection of the local anesthesia can be seen in Fig. 2.

The onset (Fig. 2, *b*) of anesthesia was recorded as the first or primary change in the action potential. In this report the onset of complete anesthesia (Fig. 2, *c*) was established as that point where there was no further indication of fiber response. Duration of complete anesthesia was measured from the onset of complete anesthesia (Fig. 2, *c*) to the initial return of nerve response to stimulation (Fig. 2, *d*). That period of time measured from the onset (Fig. 2, *c*) of complete anesthesia to the complete return of fiber response (Fig. 2, *e*) was designated as the duration of anesthesia.

Onset.—The four solutions, 1 and 2 per cent lidocaine hydrochloride and 1 and 2 per cent procaine hydrochloride, produced onset of anesthesia in less than 30 seconds after injection was begun in all experiments in which anesthesia was obtained. Anesthesia was not obtained in 2 animals (Table I). The time of onset is easily influenced and can be altered by changing the time used in the injection procedure. If the drug is infiltrated slowly, the onset of anesthesia is delayed. If injection time is brief, the first onset of anesthesia occurs sooner.

The *onset of complete anesthesia* was hastened by an increase in the volume of solution deposited at the region of stimulation. This was found to be consistent for the four solutions used (Table I). When 2 minims of 2 per cent solution of lidocaine hydrochloride were injected, the onset of complete anesthesia occurred in an average of 100 seconds after the injection was begun; with 4 minims, it occurred in 85 seconds, and with 8 minims in 58 seconds.

Elimination of the nerve response, that is, the onset of complete anesthesia, was more rapid with lidocaine hydrochloride than with procaine hydrochloride, particularly in volumes of 2 and 4 minims. Two minims of 1 per cent procaine hydrochloride did not produce a change in the action potential, whereas 2 minims of 2 per cent procaine hydrochloride produced some degree of anesthesia.

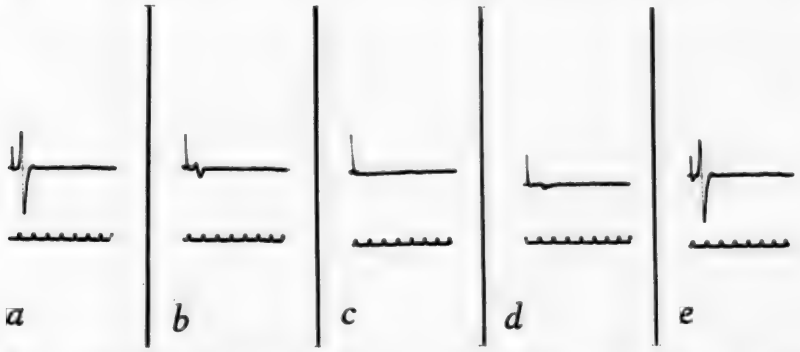

Fig. 2.—Recordings from an experiment. *a*, The activity of the nerve on application of the stimulus before the anesthetic is injected. *b*, The first alteration in nerve activity in response to the stimulus is recorded immediately after injection. *c*, Only the artifact remains; there is no nervous conduction. *d*, The initial return of activity can be seen. *e*, Complete return of nerve conduction is seen. The anesthetic has lost its effect.

TABLE I
ONSET OF COMPLETE ANESTHESIA IN SECONDS AFTER INJECTION WAS BEGUN

QUANTITY (MINIMS)	2 PER CENT LIDOCAINE HYDROCHLORIDE	1 PER CENT LIDOCAINE HYDROCHLORIDE	2 PER CENT PROCAINE HYDROCHLORIDE	1 PER CENT PROCAINE HYDROCHLORIDE
	(1)* 120	(4) 125	(6) Incomplete anesthesia	(8) No anesthesia
2	(2) 100 (3) 80	(5) 130	(7) 240	(9) No anesthesia
4	(10) 80 (11) 90	(12) 90 (13) 90	(14) 120 (15) 105	(16) 180 (17) 170 (18) 190
8	(19) 60 (20) 60 (21) 50	(22) 60 (23) 75	(24) 85 (25) 75	(26) 80 (27) 80

*Numbers in parentheses represent experiment and animal numbers.

In Animal 7, which received 2 minims of 2 per cent procaine hydrochloride, complete anesthesia was obtained in 240 seconds; however, partial return of the action potential occurred in 1 minute. Anesthesia was classified as incomplete in Animal 6.

Onset of complete anesthesia was more rapid with 4 minims of 2 per cent procaine hydrochloride than with 4 minims of 1 per cent procaine hydrochloride. With 8 minims of each of the solutions of procaine, onset of complete anesthesia was approximately the same. The duration of anesthesia, however, was greater after injection of the 2 per cent solution of procaine hydrochloride.

Return of Nerve Activity and Duration of Anesthesia.—After the onset of complete anesthesia, the next phenomenon that is expected is the *initial return* of fiber response. The nerve does not begin to conduct again in its entirety; however, there is an indication that some fibers are beginning to respond again (Fig. 2, *d*). In the interval from onset of complete anesthesia to initial return one would expect no perception of a sensory stimulus. We have elected to classify this period as *complete anesthesia.*

Two minims of 2 per cent solution of lidocaine hydrochloride yielded complete anesthesia for a longer period than 2 minims of the other solutions (Table II). This can best be appreciated if effects of the 2 per cent and the 1 per cent solutions of lidocaine hydrochloride are compared. Lidocaine hydrochloride in 2 per cent solution produced complete anesthesia for an average of 65 minutes, and in 1 per cent solution for an average of 10 minutes.

TABLE II
DURATION OF COMPLETE ANESTHESIA

QUANTITY (MINIMS)	2 PER CENT LIDOCAINE HYDROCHLORIDE			1 PER CENT LIDOCAINE HYDROCHLORIDE			2 PER CENT PROCAINE HYDROCHLORIDE			1 PER CENT PROCAINE HYDROCHLORIDE		
	MIN.		SEC.	MIN.		SEC.	MIN.		SEC.	MIN.		SEC.
2	(1)*	78		(4)	11	55	(6)	Incomplete anesthesia		(8)	No anesthesia	
	(2)	58	20	(5)	9	50						
	(3)	58	40				(7)	1		(9)	No anesthesia	
4	(10)	68	40	(12)	58	30	(14)	38		(16)	18	
	(11)	73	30	(13)	53	30	(15)	38	15	(17)	20	10
										(18)	20	50
8	(19)	119		(22)	99		(24)	68	35	(26)	58	40
	(20)	109		(23)	88	45	(25)	78	45	(27)	68	40
	(21)	119	10									

*Numbers in parentheses are the experiment and animal numbers.

The duration (the time elapsed from complete loss to complete return of nerve conduction) of anesthesia produced with 2 minims of 2 per cent lidocaine hydrochloride was found to be two to three times greater than that produced by the same volume of 1 per cent lidocaine hydrochloride or 2 per cent procaine hydrochloride (Table III). Lidocaine hydrochloride in 2 per cent solution produced anesthesia for an average of 122 minutes and in 1 per cent solution for an average of 50 minutes. In Animal 7, the duration of anesthesia from procaine hydrochloride in 2 per cent solution was 50 minutes. No anesthesia was obtained with 2 minims of 1 per cent procaine.

In the group given 4 minims, 2 per cent solution of lidocaine hydrochloride produced the longest duration of anesthesia. This drug gave an

average duration of 137½ minutes. Lidocaine hydrochloride in 1 per cent solution produced anesthesia for 110 minutes. Procaine in 2 per cent solution gave an average duration of 80 minutes, while procaine in 1 per cent solution

TABLE III
DURATION OF ANESTHESIA IN MINUTES

QUANTITY (MINIMS)	2 PER CENT LIDOCAINE HYDROCHLORIDE	1 PER CENT LIDOCAINE HYDROCHLORIDE	2 PER CENT PROCAINE HYDROCHLORIDE	1 PER CENT PROCAINE HYDROCHLORIDE
2	(1)* 130 (2) 125 (3) 110	(4) 45 (5) 55	(6) 50† (7) 50	(8) No anesthesia (9) No anesthesia
4	(10) 140 (11) 135	(12) 110 (13) 110	(14) 75 (15) 85	(16) 45 (17) 45 (18) 45
8	(19) 175 (20) 200 (21) 200	(22) 190 (23) 190	(24) 150 (25) 150	(26) 75 (27) 100

*Numbers in parentheses represent experiment and animal numbers.
†Incomplete anesthesia; however, there was some loss of response, and it took 50 minutes for nerve activity to return to normal.

gave an average duration of 45 minutes. Lidocaine hydrochloride in this volume produced anesthesia of greater duration than procaine.

With 4 minims of 1 per cent procaine hydrochloride, the duration of anesthesia was consistently 45 minutes. Other doses also gave rather consistent results (Table III). This consistency obviously may not be easily reproduced by this or any other method, but is most encouraging and justifies further investigation of this promising procedure.

Duration of anesthesia was markedly increased when 8 minims of solution were used, but it was again less with 8 minims of procaine hydrochloride than with 8 minims of lidocaine hydrochloride.

It is evident that the method employed is capable of showing the difference between the activity of one anesthetic agent over another.

COMMENT

We believe that our neurophysiologic method is adapted to evaluating the action of local anesthetics.

Our study indicated that the method is objective; personal judgment was not involved and no blind testing was required to offset the unintentional bias of the observer. The interference produced by local anesthetics in the action potential of the sensory terminal fibers of the maxillary division of the trigeminal nerve was recorded on a qualitative and quantitative basis. The electrical stimulus was consistent and could be quantitated. The animal did not accommodate to the repeated electrical stimuli.

The criterion of response was definite and readily measurable. Our study indicated also that the response was not influenced by the emotional state of the animal or a variation in the sensory threshold.

An obvious disadvantage is that all animals have to be killed.

An obvious improvement or requirement of this method would be the evaluation of the action potential which would specify whether the response of the nerve fiber is compatible with the time required for conduction of the sensation of pain and temperature or if, in reality, what we observed on the oscilloscope were only responses of touch fibers.

We used procaine for our tests because it serves as a standard for many investigators, and lidocaine hydrochloride because we were reasonably familiar with its action and because it is a drug commonly used in dental practice.

Concentration and volume of the agent used had a direct effect on the parameters of local anesthesia. The relationship of concentration, volume, and the mode of action, however, was not a linear one.

A question might be raised regarding the fact that 2 minims of 1 per cent procaine hydrochloride did not produce a demonstrable change in the action potential. Clinically, 2 drops of an anesthetic injected subgingivally would produce a topical anesthetic effect at least, but as indicated in this experiment the degree of anesthesia would be minor, and only a few nerve endings would be influenced. It certainly would not be sufficient for any dental procedure.

SUMMARY

A neurophysiologic method was designed for study of the four parameters of local anesthesia, namely (1) onset, (2) onset of complete anesthesia, (3) initial return of nerve activity, and (4) complete return of sensory nerve activity, and was tested on 27 cats. The latter were prepared by insertion of recording electrodes into the left gasserian ganglion and two stimulating needle electrodes into the left maxillary canine fossa. Alterations in the action potential of the nerve were recorded on a quantitative and qualitative basis.

Procaine hydrochloride and lidocaine hydrochloride in 1 and 2 per cent solutions and in doses of 2, 4, and 8 minims were the test solutions. These were injected 1 cm. posterior to the stimulating electrodes.

The onset of complete anesthesia was found to be hastened with an increase in volume of solution injected. This was true for both procaine hydrochloride and lidocaine hydrochloride. The onset of complete anesthesia, however, was generally found to be more rapid with lidocaine hydrochloride than with procaine hydrochloride.

Lidocaine hydrochloride in 2 per cent solution produced complete anesthesia for the longest period. This was true of doses of 2, 4, and 8 minims. The duration of anesthesia was greatest also when a 2 per cent solution of lidocaine hydrochloride was used. It was two to three times greater than with 1 per cent solution of lidocaine hydrochloride or with 2 per cent solution of procaine hydrochloride. An increase in concentration and volume resulted in greater duration of anesthesia.

Two per cent procaine hydrochloride was more effective than 1 per cent procaine hydrochloride. A finding of considerable interest was that 1 per cent lidocaine hydrochloride was more efficient anesthetically than 2 per cent procaine hydrochloride.

This study indicated that the neurophysiologic method employed is adapted to the objective evaluation of the action of local anesthetics.

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INHIBITION OF GLYCOLYSIS BY HALOGENATED COMPOUNDS

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THIS presentation is concerned with one type of organic structure which has been selected from an empirical program dealing with a property of more than 3,500 chemicals. This property is the ability of solutions of the chemicals to inhibit glycolysis in salivary sediment. It has been used to select groups of compounds which deserve further investigation as topical agents for control of dental caries.

This experimental method has been described and its precision tested.[1, 2] Findings among ethers, esters, and hydroxyl derivatives,[3] amines,[4] amides, ureas, urethane, and urea derivatives,[5] aldehydes, ketones, and organic acids[6] have been published. There were chemicals among each of these groups capable of inhibiting glycolysis.

There is one obvious requirement for inhibitory action by a chemical in this system. The chemical must be sufficiently soluble in water or mixed solvent to diffuse into the thin layer of salivary sediment and exert its effect. Many of the amides, amines, alcohols, and phenols possess sufficient water solubility because of the solubilizing action of the functional group. Halogenation does not generally make a hydrocarbon water soluble, and compounds containing no other functional groups are insufficiently soluble to exert inhibitory action. The inhibitory action of halogenation must be studied on compounds which contain some other functional hydrophilic group. The presence of two functional groups makes interpretation complicated. For example, with a halogenated phenol, it is a question initially of whether the activity is attributable to the halogen or to the hydroxyl group. Interpretation of findings with the halogenated derivatives was therefore delayed until information had been obtained regarding probable effects of other functional groups. At present, some estimate can be made as to whether the inhibitory action is attributable to halogen or to a solubilizing grouping.

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Tests are available on approximately 490 halogen derivatives, some in more than one solvent. These included 419 tests on chlorine derivatives, 21 on fluorine derivatives, and 97 on bromide and/or iodine derivatives. This group of compounds seemed to be sufficient in number to permit an evaluation of whether halogenation has any general effect on the property of glycolysis inhibition.

EXPERIMENTAL PROCEDURE

The equipment and experimental technic have been thoroughly presented elsewhere.[1, 2] A thin film of salivary sediment is coated on the surface of a glass electrode. The test chemical in aqueous solution or in 10 per cent propylene glycol is placed in contact with the film of sediment after the sediment has attained a steady state pH differential during contact with a control solution. After 20 minutes' contact, the experimental solution is replaced by a second control solution and the new differential observed. In order for a test chemical to produce inhibition of glycolysis, it must diffuse through a thin layer of salivary sediment, inactivate the organisms or the mechanisms by which the organisms convert glucose to acid, and have sufficient persistence of effect to resist rinsing by the second control solution.

RESULTS AND DISCUSSION

The question of the general effect of halogenation upon inhibitory action was approached by selecting all monochlor derivatives whose parent structures were tested in the same solvent at similar concentrations. In most instances the compounds were insufficiently soluble for test at 1 per cent, the standard concentration employed, and were studied at half saturation. The effect of halogenation can best be disclosed by comparing pairs of compounds, wherein either parent, halogen derivative, or both, are inhibitory. These data are presented in Table I. The names of the 12 parent structures are presented in the first column. The test concentration and the solvent are presented in the second column. The letters "pg" indicate that the solvent was 10 per cent propylene glycol and 90 per cent water. The values in the third and fourth columns are the percentage of recoveries with the initial control value equal to 100 per cent. These values are calculated from the steady state of pH's of sediment (pH_S) and buffer (pH_B) before and after treatment of the sediment with test chemical. Readings after treatment are marked with primes (pH_S' and pH_B') and the formula is as follows:

$$\text{Percentage recovery} = \frac{100\ (pH_B - pH_S)}{pH_B\ pH_S}.$$

The average effect of chlorination seems to be insignificant, but the wide variation in values for parent and halogenated derivatives indicate that the influence of halogenation is unpredictable rather than nil. Chlorination decreases the recovery as much as 80 per cent and increases it in another instance as much as 63 per cent. Apparently chlorination has a highly specific

action and no generality can be drawn regarding its probable action when the halogen is added to a structure which has some potentiality for inhibitory effects.

TABLE I

COMPARISON OF INHIBITORY ACTION OF MONOCHLORO DERIVATIVES AND PARENT STRUCTURES AMONG BIOLOGICALLY ACTIVE COMPOUNDS

CHEMICAL	CONCENTRATION	PERCENTAGE RECOVERY		
		PARENT	MONOCHLORO DERIVATIVE	DIFFERENCE
Phenol	1.0 pg*	0	11	11
3, 5-Dimethyl phenyl "cellosolve"	S/2 pg	11	62	51
N-Diphenyl methyl-N′-methyl piperazine	S/10 pg	19	8	-11
3-Phenyl-1-propanol	S/2 pg	30	73(o)	43
			86(p)	56
Thymol	S/2 pg	33	79	46
Thionyl aniline	S/2 pg	35	53(o)	18
			68(m)	33
2-Cyclohexyl phenol	S/2 pg	49	79	30
α, α-Dimethylphenethyl alcohol	S/2 pg	52	95(o)	43
			115(p)	63
Lactic acid acetate	0.6-0.7 pg	65	52	-13
Phenoxy propanol	1.0 or S/2 pg	69	18	-51
Phenoxy ethanol	1.0 pg	81	1	-80
Succinimide	1.0 or S/2 pg	92	37	-55
Average		45	49	

*pg = 10 per cent propylene glycol and 90 per cent water.

Table II is presented to disclose whether or not halogenation has any general property for initiating inhibitory action. Compounds presented in this table show little inhibitory action either in the parent structure or in the monochloro derivatives. On the average the chlorinated derivative has essentially the same inhibitory action as the parent compound. The mean for the parent compounds is 87 per cent, and that for the substituted compounds is 88 per cent. The divergence between parent and substituted compounds is much less than that observed in Table I. Seventy-six per cent of the pairs of values fall within 20 per cent, whereas, for the active chemicals reported in Table I, only 27 per cent fall within this range. This comparison indicates again that the wide divergence in Table I is attributable to specificity rather than to variance in the technic. The findings on fluorine, chlorine, bromine, and iodine derivatives of acetic acid[7] have some bearing on the question of whether halogens other than chlorine are able to affect the inhibitory action of a parent substance. Iodoacetate was active at 0.01 per cent, bromacetate at 0.1 per cent, and chloro- and fluoroacetate were inactive at 1 per cent. On the other hand, the para-substituted chloro- or bromophenol was active whereas iodophenol was not. The N-chlorosuccinamide was active, but the N-bromo derivative was not. Three halogen derivatives of a substituted piperazine were each active to about the same extent. This information is insufficient to permit a generalization.

TABLE II

COMPOUNDS WITH SLIGHT OR NO ACTIVITY
COMPARISON OF INHIBITORY ACTION OF PARENT STRUCTURES AND MONOCHLORO DERIVATIVES

CHEMICAL	CONCENTRATION	PERCENTAGE RECOVERY	
		PARENT	MONOCHLORO DERIVATIVE
Acetamide	1.0 or S/2	99	91
Acetanilide	S/2 pg*	77	86
Acetoxime N²-phenyl carbamate	S/2 pg	84	99(3ø)
			93(4ø)
p-Aminobenzoic acid	0.1	98	109
p-Aminobenzophenone	S/2 pg	92	89
Benzaldehyde	S/2 pg	74	70
Benzoic acid	0.1	111	98
Benzophenone	S/10	94	120
2-Butanone oxime-N²-phenyl carbamate	S/2 pg	97	102(3ø)
			94(4ø)
n-Butyl acetate	S/2 pg	79	59
α-Butylbenzyl alcohol	S/2 pg	108	97
p-tert-Butylphenol	S/10	104	92
α-Carbobutoxy ethyl N-phenyl carbamate	S/2 pg	89	90
α-Carbocyclohexoxyethyl N-phenyl carbamate	S/2 pg	83	80
α-Carboethoxyethyl N-phenyl carbamate	S/2 pg	77	75 (ethoxy)
			88(3ø)
α-Carboxyethyl N-phenyl carbamate	1.0 pg	77	66
Cyclohexane ethanol acetate	S/2 pg	94	72
Nitrobenzene	S/2 pg	78	85
4-Nitrophenol	S/2 pg	56	45
2-Nitropropane	S/2 pg	81	70
Phenethyl alcohol	1.0 or S/2	92	80
Phenylethyl alcohol, α-ethyl-α-methyl-	S/2 pg	102	94(o)
			92(p)
	S/2 pg	75	74
Phenetole Phenoxyacetic acid	0.1	88	112
o-Phenyl phenol	S/10	95	98
p-Phenyl phenol	1.0 or S/2 pg	95	95
Propionic acid	0.1	90	111
	S/2 or 1.0 pg	91	112
	S/2 or 1.0 pg	82	91
Propiophenone	S/2 pg	83	101
Propynyl N-phenyl carbamate	S/2 pg	79	81
Quinoxalinium iodide, 1-methyl-	S/2 pg	84	73
Salicylaldehyde	S/2 pg	67	78
Xanthine	S/2 pg	89	90
Average		87	88

*pg = 10 per cent propylene glycol and 90 per cent water.

Next, a comparison was made between compounds containing two or more halogens and those containing one or none, in order to determine whether compounds having multiple halogenation are generally similar to parent structures. There were available for comparison 34 pairs of values where a recovery figure was available on a compound containing two chlorine atoms; for comparison, data obtained on a parent structure were used if it were available, otherwise data on the monochloro derivative were employed. The values on a parent or monosubstituted compounds were subtracted from those obtained from the disubstituted compound. The average difference was 13 ± 27 per cent. On the average the disubstituted compounds had slightly less activity than the monosubstituted or parent structures, but the high standard deviation of a single determination shows that this mean cannot be

trusted as a generalization. The effect is highly specific, for the difference varies from –28 to +66 per cent. Among trichlorinated derivatives there were 15 comparisons possible, showing a mean differential of 3 ± 24 per cent, with a range from –46 to +32. Once again there is evidence that the influence is highly specific, even though trisubstitution increased inhibition as often as it decreased it. Tetrasubstituted compounds were few and only 7 comparisons could be made; in every case less inhibition occurred with the compounds containing four chlorine atoms. The mean effect was 38 ± 47 per cent, and the range was from 7 to 92 per cent increase in recovery.

The complete series of compounds tested is presented in Table III, in which they are grouped according to decreasing activity range and to the solvents used. Within any given group the compounds are arranged alphabetically according to one basic chemical grouping. This table of compounds is presented in order to permit comparisons between active and inactive structures, and to list the considerable numbers of structures which have been found ineffective. Some of the compounds have been listed before.[6] Among the most active substances tried in water solution (Table III, Sec. 1, a) are a wide variety of structures. Iodoacetamide may owe its activity to its close chemical similarity to iodoacetate, one of the most active chemicals tried. The ethylene diamine derivative is of a type known to be inhibitory,[4] the halogenated phenols show no apparent advantage over phenol itself,[3] and the substituted piperazines would probably be active in case the halogens were not present.[4] Since the phenoxyethanol itself is not inhibitory, it appears that the ortho and parachloro substitution on this compound brings about inhibitory action. Mucochloric acid possesses an unusual structure which prevents comparison with our existing list of compounds. Presumably the inhibitory action of N-chlorosuccinimide can be attributable to its oxidizing action, since N-bromosuccinimide is less inhibitory and succinimide itself is inactive.

TABLE III*
HALOGEN TABLE

I. Recovery of 0 to 19 per cent

a. Tested in water solution

Acetamide, iodo-*
Acetic acid, bromo-*
Ethanol, o-chlorophenoxy-2-
Ethanol, p-chlorophenoxy-2-*
Ethylene diamine, chloro-α-methylbenzyl-**
Mucochloric acid**
Phenol, o-chloro-**
Phenol, 2, 4, 5-trichloro-**
Piperazine, N-(p-bromodiphenylmethyl)-N′-methyl-, hydrochloride**
Piperazine, N-(m-chlorodiphenylmethyl)-N′-methyl-, hydrochloride**
Piperazine, N-(p-fluorodiphenylmethyl)-N′-methyl-, hydrochloride**
Rose bengal*
Succinimide, N-chloro-**

b. Tested in 10 per cent propylene glycol solution

Benzyl alcohol, 3, 4-dichloro-α-methyl-
Ethanol, p-chlorophenoxy-2-*
Phenol, p-bromo-*
Phenol, p-chloro-*
Phenol, p-fluoro-*
Phenol, 2, 2′ methylene bis(p chlorophenol)**
Phenol, 4 and 6-chloro-2-phenyl**
Propane, 1, 1-dichloro-1-nitro-**
Propanol, 1-(p-chlorophenoxy)-2-
Propionic acid, α-bromo-*

*For footnotes to Table III, see page 366.

II. Recovery of 20 to 39 per cent

a. Tested in water solution
- Acetaldehyde, chloro-**
- Acetic acid, bromo-**
- Hydantoin, 1, 3-dichloro-5, 5-dimethyl-**
- Mucochloric acid
- Phenol, 4, 5-dichloro-2-iodo-**
- Piperazine, N-(o-chlorodiphenylmethyl)-, N'-methyl-hydrochloride**(1)
- Propionic acid, α-bromo-*
- Saligenin, 3, 5, 6-trichloro-**

b. Tested in 10 per cent propylene glycol solution
- Acetic acid, 2 butyl-x-chlorophenoxy-*
- Aniline, thionyl-
- Benzyl alcohol, 3, 4-dichloro-
- Butyric acid, α-bromo-γ-hydroxy-γ-lactone
- Cadaverine, N-(1-chlorophenyl)-N'-isopropyl-, monohydrochloride**
- Ethanol, 2-{[4-(7-chloro-4-quinoliamino) pentyl]ethylamino}-, monosulfate
- Ethanol, o-chlorophenoxy-2-*(3)
- Ethanol, 3, 4-dichlorophenoxy-2-(3)
- Methanol, 3, 4-dichloro-1-
- Phenol, 2-bromo-4, 5-dichloro-**
- Phenol, 2, 4-dichloro-
- Phenol, 4, 5-dichloro-2-iodo-
- Phenol, 2, 4, 5-trichloro-
- Phenol, 2, 4, 6-trichloro-
- Propane, 1-chloro-1-nitro-
- Salicylic acid, 3, 5-diiodo-
- Succinimide, N-chloro-
- Urea, o-chlorobenzyl isothio-*

c. Tested in 10 per cent triethylene glycol (Dowanol 62B)
- Ethanol, 3, 4-dichlorophenoxy-2-**
- Propanol, 1-(o-chlorophenoxy)-2-*

III. Recovery of 40 to 59 per cent

a. Tested in water solution
- Acetamide, chloro-**
- Butyric acid, perfluoro-**
- Chloranil**
- Ether, bis-β-chloroethyl-**
- Phenol, o-chloro-*
- Quinoline, chlorohydroxymethyl-**

b. Tested in 10 per cent propylene glycol solution
- Acetate, n-butyl chloro-
- Acetate, lactic acid chlorol*
- Acetate, 5-iodo salicylic acid*(3)
- Aniline, o-chloro thionyl
- Carbamate, ammonium N-p-chloro-phenyl dithio-
- Carbamate, diethylene glycol bis (N-3-chlorophenyl)-
- o-Cresol), 6, 6'-thio(4-chloro-α-dimethyl-amino-
- Ethanol, 4-chloro-3, 5-dimethylphenoxy-2-
- Ethanol, o-chlorophenoxy-2-
- Ethanol, 3, 4-dichlorophenoxy-2-**
- Phenol, 2-chloro-4-nitro-
- Phenol, 2 and 4-chloro-6-phenyl-**
- Phenol, 2, 3, 5, 6-tetrachloro-
- Phenol, tetrachlorobutyl-, mixed
- Phenol, 2, 3, 5, 6-tetrachloro nitro-
- Phenol, 2, 4, 6-trichloro-**
- Salicylic acid, 3, 5-diiodo-(4)
- Saligenin, 3, 5, 6-trichloro-**
- Sulfide, [(2, 3, or 4-chloro-α-methylbenzyl) 2-hydroxyethyl]-
- m-Xylenol, p-chloro-

c. Tested in 10 per cent triethylene glycol (Dowanol 62B)
- Acetanilide, p-bromo-
- Acrylic acid, 3-(p-chlorobenzyl)-
- Anisole, o-chloro-
- Phenetole, p-chloro-**

IV. Recovery of 60 to 79 per cent

a. Tested in water solution
- Acetate, n-butyl-2, 4, 5-trichloro-*
- Acetic acid, o-chlorophenoxy**
- Acetic acid, 2, 4-dichlorophenoxy**
- Aniline, p-bromo-, hydrochloride
- Butanedione, 4, 4, 4-trifluoro-1-(2-thienyl-1, 3-**
- Carbamate, isopropyl N-3-chlorophenyl-**
- o-Cresol, 4-(7-chloro-4-quinolylamino-α-(1-methylallylamino)-dihydrochloride-1¾ hydrate
- Cyclohexanol, 2-chloro-**
- Ethanol, 2-bromo-
- Ethylamine, 2-bromo-, hydrobromide*
- Ethyl sulfate, 2-chloro-4-dodecyl phenoxy-2-**
- Ethyl sulfate, pentachlorophenoxy-2-**
- Hydroquinone, x, 2-dichloro-
- Phenol, 2-brom-4-phenyl-**
- Phenol, 4-chloro-2-cyclohexyl-
- Phenol, p-iodo-
- cis-Phthalic acid, hexachloroendomethylene-tetrahydro-**
- Piperazine, 1, 4-(3, 5-dichloro-2-hydroxy benzyl)(1)
- Propanol, 2, 2, 3, 3-tetrafluoro-1-**
- Propionate, ethyl-α-dibromo-**
- Propionic acid, α-bromo-*
- Propionic acid, 2-iodo-**
- Salicylic acid, 3, 5-diiodo-
- Saligenin, 3, 5, 6-trichloro-
- Toluidine, 4-chloro-2-

b. Tested in 10 per cent propylene glycol solution
- Acetamide, chloro-
- Acetanilide, p-iodo-
- Acetate, benzyl-chloro
- Dimethanohephthalene, 1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 8, 8a-hexahydro-1, 4-endo, endo-5, 8-

Acetate, 2-cyclohexylethyl chloro-
Acetic acid, (2, 3, 5, 6-tetrachlorophenoxy-
Aniline, p-bromo-
Aniline, N-2, 4-dichlorobenzylidene-3-chloro-
Aniline, 3-chloro-4-phenoxy-
Aniline, m-chloro thionyl-
Anisole, o-bromo-
Anisole, 3-iodo-5-nitro-
Benzaldehyde, o-chloro-
Benzaldehyde, bis (carboxymethyl)-mercaptal 2, 4-dichloro-**
Benzaldehyde, 3, 4-dichloro-
Benzene, 1-bromoethyl-**
Benzene, 1-bromo-1-nitro-
Benzene, 3, 4-dichloronitro-
Benzene, pentachloronitro-
Benzene, 2, 4, 5-trichloronitro-
Benzoic acid, 2-amino-3, 5-diiodo-*
Benzoic acid, 3-amino-5-iodo-
Benzonitrile, p-chloro-
Benzyl alcohol, 2, 4-dichloro-α-methyl-**
Butyrate, 2, 4-dichlorophenoxy-2-ethyl-
Carbamate, 3-amyl N-3-chlorophenyl-
Carbamate, sec-butyl N-3-chlorophenyl-
Carbamate, tert-butyl N-3-chlorophenyl-
Carbamate, α-carbo-(2-chloroethoxy)ethyl N-phenyl-
Carbamate, α-carboxy ethyl N-3-chlorophenyl-*
Carbamate, 2-chloroethyl N-phenyl-
Carbamate, 2-chloroethyl N-2-methyl-5-chlorophenyl-
Carbamate, 2-(10 chloro-3-isopropoxy) propyl N-phenyl-
Carbamate, β-2, 4-dichlorophenoxy ethyl N-3-chlorophenyl-
Carbamate, 2-(1, 3-dichloro) propyl N-phenyl-
Carbamate, ethylene glycol bis-(N-3-chlorophenyl)-
Carbamate, 4-indanyl N-3 chlorophenyl-
Carbamate, 5-indanyl N-3 chlorophenyl-
Carbamate, isopropyl N-3-chloro-4-methyl-phenyl-
Carbamate, isopropyl N-2-(5-chloro) pyridyl-
Carbamate, isopropyl N-2-methoxy-5-chlorophenyl-
Carbamate 1-(2-methyl) butyl N-3-chlorophenyl-
Carbamate, α-phenylethyl N-3-chlorophenyl-
Carbanilic acid, m-chloro-, lactonitrile ester
Carbazate, 2-chloroethyl N^2-phenyl-
Carbazate, isopropyl N^2-2, 5-dichlorophenyl-
Carbonate, N-butyl chloro-
Carbonate, diethyleneglycol bis [1-carbo-2(2-chloroethoxy)ethoxy ethyl]-*
Ethane, 1-(2-chloroethoxy)-2-(p-chlorophenoxy)-
Ethanol, o-chlorophenoxy-2-
Ethyl sulfate, 4-chloro-3, 5-dimethylphenoxy-2-
Ethanol, 3, 5-dichloro-2-biphenyloxy-2-
Ether, dipropylene glycol-o-chlorophenyl-*
Ether, dipropylene glycol-2, 4-dichlorophenyl-
Formate, diethylene glycol chloro-
Maleate, di(chloroethyl)chloro-**
Malonic acid, (m-chlorophenethyl)-**
Phenetole, 4-tert-butyl-β, 2-dichloro-
Phenetole, p-chloro-
Phenol, 2-sec-butyl-4-chloro-
Phenol, 2-chloro-4-cyclohexyl-
Phenol, 4-chloro-2-cyclohexyl-
Phenol, 4, 5-dichloro-2-iodo-**
Phenol, p-iodo-**
Phenol, 2, 2′-thio bis 4-6-dichloro-
Phthalimide, N-bromo-
Piperazine, N-(p-chlorodiphenyl methyl)-N′-methyl-, hydrochloride*
Propane, 2-chloro-2-nitro-
Propanediol, 3-chloro-1, 2-*
Propanol, 3-(o-chlorophenyl)-1-
Propanol, 1-(2, 4-dichlorophenoxy)-2-
Propanol, 1-(2, 3, 4, 6-tetrachlorophenoxy)-
Propiophenone, 3-chloro-4′-hydroxy-3′ methoxy-
Pyridine, 3-bromo-*
Quinoline, 7-chloro-4-(4-morpholino cyclo hexylamino)-
Quinoline methanol, 7-chloro-α-(diethyl aminoethyl)-2-phenyl-4-, hydrochloride
Quinoxalinium iodide, 6(or 7)-chloro-1-methyl-
Rhodanine, 3-(4-chlorophenyl)-5(4-chlorobenzilidine)-
Rhodanine, 3-(4-chlorophenyl)-5-furfurylidine-
Salicylaldehyde, 5-chloro-
Salicylaldehyde, 3, 5-dibromo-
Salicylic acid, 5-bromo-
Semicarbazone, thio-, 2, 4-dichlorobenzaldehyde
Succinimide, N-bromo-
Sulfanilamide, N^4-[(p-chlorophenyl-amidino)amidino]-N-2-pyrazinyl-, hydrochloride
Thiazolidene, 2-trichloromethyl-3-(p-chlorophenyl)-4-
Thymol, 6-chloro-
Toluene, m-chloro-
Urea, thio-, allyl-3-chlorophenyl-
Xylenol, 4-chloro-3, 5-
Xylenol, 2, 4-dichloro-3, 5-
Xylenol, 2, 4, 6-trichloro-3, 5-

c. Tested in 10 per cent triethylene glycol (Dowanol 62B)

Benzyl alcohol, p-chloro-α-methyl-**
Butyric acid, α, β-dibromo-**
Salicylic acid acetate, 5-bromo-

V. Recovery of 80 to 99 per cent

a. Tested in water solution

Acetamide, chloro-
Octanoic acid, perfluoro-**

Acetate, butoxyethoxypropanol-2, 4-dichlorophenoxy-*
Acetate, butoxyethoxypropanol-2, 4, 5-trichlorophenoxy-*
Acetate, fluoro-*
Acetate, trichlorophenyl-**
Acetic acid, chloro-
Acetic acid, 4-chloro-o-toloxy-
Acetic acid, trichloro-*
Acetic acid, 2, 4, 5-trichlorophenoxy-**
Acetic acid, trifluoro-**
Acetophenone, p-chloro-**
Acetophenone, α-chloroisonitroso-**
Adipic acid, perfluoro-**
Aniline, o-chloro-**
Barbituric acid, 5, 5-dibromo-**
Benzene hexachloride**
Benzidene, dichloro-, sulfate**
Benzoic acid, p-chloro-**
Benzophenone, p-bromo-**
Butanol, 1, 1-dihydroperfluoro-**
Butyric acid, β-bromo-*
Butyric acid, 2-bromo-3-hydroxy-*
Camphor, 3-bromo-**
Caproic acid, α-bromo-ε-benzamino-**
Carbamate, isopropyl N-β-chloroethyl-**
Carbonate, bis (p-chlorophenyl)-
Chloramine T**
Chloranilic acid**
Diethylamine, 2, 2'bis(p-chlorophenoxy)-N-methyl-
Ethane, 1-(2-biphenylyloxy)-2-(2-chloroethoxy)-**
Ethanol, 2, 4-dichlorophenoxy-2-sulfoacetate*
Ethanol, (pentachlorophenoxy)-2-**
Ethylene, 1, 2-bis(p-methoxyphenyl)-1-(p-guanylphenyl)-chloro-
Ethylene, tris (p-anisyl)-
Furoic acid, 5-bromo-2-
Furoic acid, 5-chloro-2-
Hexachlorophene**
Hydroquinone, chloro-**
p, p'-Isopropylidene, bis[2-(2, 6-dichlorophenoxy)-, disulfate]**
Morpholine, N-3-[2-chloro-4-phenylphenoxy] propyloxazine-1, 4-, hydrochloride**
Pentane, 2-bromo-*
Pentanol, 2, 2, 3, 3, 4, 4, 5, 5-octafluoro-1-**
Phenethyl alcohol, p-chloro-
Phenetole, β-chloro-*
Phenetole, β-chloro-o-phenyl-**
Phenol, p-bromo-**
Phenol, 2-bromo-4-tert-butyl-**
Phenol, 2-sec-butyl-4, 6-dichloro-**
Phenol, N-tert-butyl-2-chloro-**
Phenol, 2-chloro-4-cyclohexyl-
Phenol, 2-chloro-4, 6-dinitro-**
Phenol, 2-chloro-6-phenyl-**
Phenol, 2, 4-dichloro-**
Phenol), 2, 2'-methylene bis(p-chloro-**
Phenol), 2, 2'-methylene bis(4-chloro-6-isopropyl-**
Phenol, 1-hydroxyethyl-2, 4-dichloro-6-sulfonate-*
Phenol), 2, 2'-thio bis(4, 6-dichloro-**
Phenol, 2, 4, 6-tribromo-**
Phenothioxin, x-chloro-**
Phthalic anhydride, tetrachloro-*
Piperazine, 1-carbethoxy-4-[2-(5-chloropyrimidyl)]-**
Piperazine, N-(p-chlorodiphenylmethyl)-N'-butyl-, hydrobromide**
Piperazine, N-(p-chlorodiphenylmethyl)-N'-methyl-, hydrochloride**(2)
Piperazine, N-methyl-N'-(p-chlorodiphenylmethyl)-2-keto-, dioxalic acid**
Propanol, 1-chloro-2-**
Propanol, 1, 1, 1-tribromo-2-methyl-**
Propionate, p-chlorophenyl-
Propionate, methyl-α-chloro-**
Propionic acid, β-chloro-α-amino-*
Propionic acid, α-(o-chlorophenoxy)-**
Propionic acid, 2, 2-dichloro-*
Propionic acid, β-iodo-*
Propionic acid, perfluoro-**
Pyridinium chloride, 1-(2, 4-dichlorobenzyl)-**
Salicylaldehyde, 3, 5-dibromo-
Succinimide-, N-bromo-**
Succinimide, -N-chloro-
m-Xylenol, p-chloro-**

b. Tested in 10 per cent propylene glycol solution

Acetamide, 2, 4-dichlorophenoxy-
Acetanilide, p-bromo-
Acetanilide, p-chloro-
Acetate, 2, 4-dichlorophenoxyethyl-
Acetate, phenethyl chloro-
Acetic acid, o-chlorophenoxy-
Acetic acid, 4-chloro-o-toloxy-(3)
Acetophenone, p-bromo-
Acrolein anil, α-chloro-
Acrolein anil, α-chloro-β-anilino-, hydrochloride
Aniline, p-iodo-
o-Anisidine, 5-chloro-, hydrochloride
Anisole, o-iodo-
Anisole, 2, 3, 5, 6-tetrachloronitro-
Anisole, 2, 4, 5-trichloro-
Anthranilic acid, 5-chloro-
Carbonate, thio-, S-allylopentachlorophenyl-
Carbonate, thio-, S-ethyl pentachlorophenyl-
Cinnamide, o-chloro-N, N-diethyl-
N-Cyclopentenylamine, N-(o-chlorophenyl)-
Dimethanonaphthalene, 1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4, 5, 8-
Dimethanonaphthalene, 1, 2, 3, 4, 10, 10-hexachloro-exo-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4-endo, endo-5, 8-
Dimethanonaphthalene, 1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 8, 8a-hexahydro-1, 4, 5, 8-
m-Dioxane, 2-(o-chlorophenyl)-5-methyl-5-nitro-

Benzaldehyde, 2, 4-dichloro-
Benzaldehyde, α-hydroxy-5-bromo-
Benzaldehyde, pentachloro-
Benzamide, 2-(allyloxy)-5-bromo-N-methyl-
Benzene, 1-chloro-3-nitro-
Benzene, 1, 4-dichloro-2-nitro-
Benzene, 1-iodo-3-nitro-
Benzene, 1-iodo-4-nitro-
Benzene, 2, 3, 4, 5-tetrachloronitro-
Benzene, 2, 3, 5, 6-tetrachloronitro-
Benzenetetrol, 3, 6-dichloro-1, 2, 4, 5-**
Benzhydrol, α-tert-butyl-4, 4′-dichloro-
Benzoic acid, p-chloro-, piperonyl ester
Benzoic acid, o-iodo-
Benzophenone, 4-amino-4′-chloro-
Benzophenone, 2, 4′-dichloro-
Benzyl alcohol, α-butyl-o-chloro-
Benzyl alcohol, 2, 4-dichloro-
Benzyl alcohol, α-tert-butyl-o-chloro-
Benzyl alcohol, 2, 5-dichloro-α-methyl-**
Benzyl alcohol, 3, 4-dichloro-α-methyl-**
Butane, 1, 4-dibromo-
Buten-1-ol, 1-(o-chlorophenyl)-3-
Butyrate, 2-chloro-
Butyric acid, 2-chloro-4-hydroxy-γ-lactone
Butyric acid, 2-chloro-3-methyl-
Carbamate, acetoxime N²-3-chlorophenyl-
Carbamate, acetoxime N²-4-chlorophenyl-
Carbamate, acetoxime N²-2, 5-dichloro phenyl-
Carbamate, tert-amyl N-3-chlorophenyl-
Carbamate, 2-butanone oxime N²-4-chlorophenyl-
Carbamate, α-carbobutoxy ethyl N-3-chlorophenyl-
Carbamate, α-carbo-(2-chloroethoxy) ethyl N-3-chlorophenyl-
Carbamate, α-carbocyclohexoxyethyl N-3-chlorophenyl-
Carbamate, α-carbo-(2, 4-dichlorophenoxyethoxy) ethyl N-3-methylphenyl-
Carbamate, α-carbo-(2, 4-dichlorophenoxy) ethoxyethyl N-phenyl-
Carbamate, α-carboethoxyethyl N-3-chlorophenyl-
Carbamate, β-chloroallyl N-3-chlorophenyl-
Carbamate, γ-chloroallyl N-3-chlorophenyl-
Carbamate, 2-(1-chloro-3-butenyl) N-3-chlorophenyl-
Carbamate, 2-chloroethyl N-3-chlorophenyl-
Carbamate, 2-chloroethyl N-3-cyanophenyl-
Carbamate, 2-chloroethyl N-3, 5-dichlorophenyl-
Carbamate, 2-chloroethyl N-3-methylphenyl-
Carbamate, 2-chloroethyl N-2-methoxy-5-methylphenyl-
Carbamate, 3-chloropropyl N-phenyl-
Carbamate, β-(2-cyanoethoxy) ethyl-N-3-chlorophenyl-
m-Dioxane, 2-(p-chlorophenyl)-5-methyl-5-nitro-
Dodecan, 1-bromo-
Ethane, 1-(2-biphenylyloxy)-2-(2-chloroethoxy)-**
Ethane, -1(2-chloroethoxy)-2-(p-cyclohexyl phenoxy)-
Ethanol, 2-(p-bromophenoxy)-**
Ethanol, 2-[4-(o-chlorophenyl)phenoxy]-
Ethanol, 2-(2, 4-dichlorophenoxy)-carbonilate
Ethanol, 2-(2, 4, 5-trichlorophenoxy)-**
Formal), di-(2-chloroethyl-
Fumarate, 2-(2-chloroethoxy)ethyl-*
Fumarate, 2-chloroethyl-*
Glycine, N-[N-(α-bromopropionyl) glycyl]-
Heptanol, 2, 2, 3, 3, 4, 4, 5, 5, 6, 6, 7, 7-dodecafluoro-1-
Hexachlorophene[3]
Hippuric acid p-chloro-**
Hydroquinone, bromo-**
Hydroquinone, 2, 5-diamine-3, 6-dichloro-
Hydroquinone, dichloro-
Hydroquinone, tetrachloro-
Igepal, iodoacetate
Indole, 2-(p-chlorophenyl)-
Lactate, 2, -dichlorophenoxyethyl-
Maleate, 2-(2-chloroethoxy)ethyl-*
Penten-4-yn-3-ol, 1-chloro-3-ethyl-1-
Phenethyl alcohol, o-chloro-α-dimethyl-
Phenethyl alcohol, o-chloro-α-ethyl-α-methyl-
Phenethyl alcohol, p-chloro-α-ethyl-α-methyl-
Phenethyl alcohol, o-chloro-α-isobutyl-α-methyl-
Phenethyl alcohol, 2, 5-dichloro-**
Phenethyl alcohol, 2, 4-dichloro-α-ethyl-α-methyl-
Phenetole, 2, 3, 5, 6-tetrachloro-*
Phenol, 2-bromo-4-tert-butyl-
Phenol, 2-bromo-4-phenyl-**
Phenol, 4-sec-butyl-2-chloro-
Phenol, o-chloro-**
Phenol, o-chloro-, acetate
Phenol, p-chloro-, acetate
Phenol, 2-chloro-4-phenyl-
Phenol, 2-chloro-6-phenyl-tert
Phenol, 2, 6-dibromo-4-butyl-
Phenol, p-fluoro-**
Phenol), 2, 2′-methylene bis (p-chloro-[3]
Phenol), 2, 2′-thio bis (4-chloro-, dimethylamine salt
Phenol), 2, 2′-thio bis (4, 6-dichloro-,
Phthalanilic acid, 3′-chloro-
Propane, 1, 1-dichloro-1-nitro[2]
Propanol, 3-chloro-1-*
Propanol, 3-(p-chlorophenyl)-1-
Propanol, 2, 4-dibromo-1-
Propanol, 1-(allyloxy)-3-chloro-2-
Propanol, 1-chloro-3-isopropoxy-2-*
Propanol, 1-(2, 4, 5-trichlorophenoxy)-2-
Propionate, 2-chloroethyl α-acetoxy-*
Propionate, p-chlorophenyl-**
Propionic acid, β-chloro-*

Carbamate, cyclopenten-3-yl N-3-chlorophenyl-
Carbamate, β-2, 4-dichlorophenoxyethyl-N-phenyl-
Carbamate, 1-ethynylcyclohexyl N-3-chlorophenyl-
Carbamate, sec-isoamyl N-3-chlorophenyl-
Carbamate, isopropyl N-3, 3-dichlorophenyl-
Carbamate, isopropyl N-3, 4-dichlorophenyl-
Carbamate, isopropyl N-2, 4-dichlorophenyl-
Carbamate, isopropyl N-3-methyl-3-chlorophenyl-
Carbamate, isopropyl N-2-methyl-5-chlorophenyl-
Carbamate, propynyl N-3-methyl-5-chlorophenyl-
Carbamate, 2-(1, 3, 4-trichloro)-butyl N-phenyl-
Carbamate, 2-(1, 1, 1-trichloro-2-methyl) propyl N-3-chlorophenyl-
Carbamate, 1, 1, 1-trichloro-3-nitro-2-propyl N-phenyl-
Carbazate, isopropyl N²-2, 4, 6-trichlorophenyl-
Carbonate, allyl pentachlorophenyl-
Carbonate, isopropyl pentachlorophenyl-
Propionitrile, β-bromo-*
Pyrazole, 5-chloro-3-methyl-1-phenyl-
Pyridine, 2-bromo-*
Pyrone, 2-chloromethyl-5-hydroxy-1, 4-
Quinoline carboxylic acid, 7-chloro-4-hydroxy-3-
Quinolinium chloride, 4-chloro-2-(p-dimethylamino phenyliminomethyl) 6-methoxy-1-methyl-
Quinone, 2, 6-dichloro-
Quinoxaline, 2, 3-dichloro-
Resorcinol, 4, 6-dichloro-**
Resorcinol, 2, 4, 6-trichloro-**
Rhodanine, 3-(4-chlorophenyl)-
Salicylic acid, 3, 5-diiodo-[5]
Salicylic acid, 5-iodo-
Succinic acid, tetrafluoro-
Sulfurous acid, bis(2-chloroethyl)ester
Thiocyanate, 3, 4-dichlorobenzyl-
Toluidine, 4-chloro-2-**
m-Toluidine, 6-(ethylsulfonyl)-α, α, α-trifluoro-
Triazine, 2, 4-dichloro-6-(o-chloroanilino)-5-
Trioxane, 2, 4, 6-tris(chloromethyl)-3-
Urea, phenyl-3-chlorophenyl-
Urea, thio-, 1, 3-bis(3-chlorophenyl)-
Urea, (2, 2, 2-tribromo-1-hydroxyethyl)-
Xanthine, 8-chloro-

c. Tested in 10 per cent triethylene glycol (Dowanol 62B)

Acetanilide, p-bromo-**
Acetate, 5-iodosalicylic acid-
Benzoic acid, p-chlorophenyl ester
Hydroquinone, chloro-**
Thiazole, 2-chloro-

VI. Recovery of 100 per cent and above

a. Tested in water solution

Acetanilide, p-fluoro-**
Acetate, butyl-2, 4-dichlorophenoxy-*
Acetate, 5-iodo salicylic acid-**
Acetate, isopropyl-2, 4-dichlorophenoxy-*
Acetate, isopropyl-2, 4, 5-trichlorophenoxy-**
Acetic acid, 4-chlorophenoxy-**
Acetic acid, dichloro-**
Acetic acid, 2, 4-dichlorophenoxy-, ammonium salt**
Benzene, hexachloro-**
Benzoate, 2-diethylaminoethyl-3-amino-6-chloro-**
Benzoic acid, p-chloro-
Benzoic acid, 2-chloro-4-amino-**
Benzoic acid, p-chloro-, p-chlorophenyl ester
Benzoic acid, p-chlorophenyl ester
Benzophenone, p-chloro-**
Benzophenone, 2, 4'-dichloro-**
Chloroform**
Cyclohexadine-1-one, hexachloro-3, 5-**
Ethane, dichlorodiphenyl trichloro**
Ether, bis(2-chloroisopropyl)-**
Ether, perfluoro cyclic C8-**
Ethylene, perchloro-**
Hexanoic acid, perfluoro-**
Hydroquinone, tetrachloro-**
Lepidine, 2-chloro-**
Maleate, chloroethyl-**
Methane, bis (p-chlorophenoxy)-**
Methane, bromodiphenyl-**
Phenacyl bromide, p-phenyl-**
Phenol, 2-chloro-4-phenyl-**
Phenol, 2, 6-dichlorobenzenone indo-3'-chloro-sodium salt**
Phenol, 2, 4-dichloro-6-sulfonate-*
Phenol, pentabromo-*
Phthalimide, β-bromoethyl-**
Piperazine, N-(p-bromobenzyl)-hydrochloride**
Piperazine, N-(p-chloridiphenyl methyl)-N'-(2-thienyl methyl)-, dihydrochloride**
Proline, methyl bis (β-chloroethyl) amine-**
Propionic acid, β-chloro-
Propionic acid, α-(p-chlorophenoxy)-**
Salicylic acid, 5-bromo-**
Salicylic acid, 5-iodo-**
Stearic acid, bromo-*
Styrene dibromide**
Succinic acid, α, β-dibromo-*
Succinic acid, perfluoro-**
Sulfonate, p-chlorobenzene-, p-chlorophenyl**
Thio ether, 2-imidazolyl-p-chlorobenzyl-**
2-Thiophene sulfonyl chloride**
Thymol, 6-chloro-**
Triethylamine, 2-(2, 4, 6-triiodophenoxy)-, hydrochloride
Tyrosine, diiodo**

b. Tested in 10 per cent propylene glycol solution

Acetic acid, p-chlorophenyl-
Benzamide, o-chloro-N, N-dipropyl-
Benzoate, sucrose octa-2, 4-dichloro-
p-Benzoquinone, 2, 5-dichloro-
Benzyl alcohol, α-butyl-p-chloro-α-methyl-
Benzyl alcohol, o-chloro-α-isopropyl-
Butyric acid, 2-bromo-*
Carbamate, N-amyl N-3-chlorophenyl-
Carbamate, 2-butanone oxime-N2-3-chlorophenyl-
Carbamate, α-carbo-(2, 4-dichlorophenoxy)ethoxyethyl N-3-chlorophenyl-
Carbamate, β-N'-(phenoxy acetamido) ethyl N2-3-chlorophenyl-
Carbamate, propynyl N-3, 5-dichlorophenyl-
m-Dioxane, 2-(o-chlorophenyl)-5-ethyl-5-nitro-
m-Dioxane, 2, -(p-chlorophenyl)-5-ethyl-5-nitro-
Ethane, 1-(2-chloroethoxy)-2-(2, 4, 6-trichlorophenoxy)-
Ether, 2, 3, 5, 6-tetrachloro phenyl-n-butyl-
Imidazoline, 2-[2-(2-chloroethoxy) ethyl thio]-2, hydrochloride
Lactic acid, 2-(2, 4-dichlorophenoxy) ethanol ester
Phenethyl alcohol, α(chloromethyl)-
Phenethyl alcohol, p-chloro-α, α-dimethyl-
Phenethyl alcohol, 3, 4-dichloro-α-ethyl-α-methyl-
Phenol, 2, 4, dichloro, -acetate
Phenol, 2, 4-dichloro-6-phenyl-
Phenol, tetrachloro-2, 4'-methylene di-
Phenol, 4, 4'-(2, 2, 2-trichloroethylidene) di-
Propane, di (2, 6-dichlorophenylol)-
Propionate, methyl-β-chloro-
Propionate, phenethyl-3-bromo-
Propiophenone, p-chloro-**
Saligenin, 5-chloro-

No marks = S/2, obtained by 1:1 dilution of saturated solution of the chemical in 20 per cent propylene glycol.
* = 1 per cent.
** = 0.1 per cent or S/10.
(1) Inactive by another series of tests.
(2) Active by another series of tests.
(3) Tested in 20 per cent propylene glycol.
(4) Fifty-minute contact.
(5) Twenty-minute contact.

Fig. 1 presents examples of inhibitory halogenated compounds, selected in part to be representative of instances in which the halogen seems to bring about or augment activity. For example, the first three compounds are acetic acid derivatives and acetic acid is not inhibitory. The bromine derivative imparts inhibitory action to propionic acid, and chlorination produces an active chemical from acetaldehyde. The remaining three chemicals in the first column seem to owe their activity to a combination between nitro and halogen groups; 2-nitropropane is inactive but the 2-nitrochloropropane falls among the active chemicals. In the second column of Fig. 1 is phenol and related structures. The first three compounds are phenols and none are as active as phenol itself. The fourth compound is a dichloro derivative of benzyl alcohol, which is somewhat more active than benzyl alcohol. The inactivity of the 2, 4-dichloro derivative is surprising. The α-methyl derivative of 3, 4-dichloro benzyl alcohol is active. These findings indicate that both the number and positions of halogens have a considerable effect on the activity of benzyl alcohol. The last compounds, 2, 3, 4-trichlorocyclohexyl methanol, is similar in structure to the disubstituted active alcohol. There is indication that the activity seen with phenol will persist when the hydroxyl group is still further removed from the benzene ring. This holds for ortho and parachlorophenoxy ethanol, and for the parachlorophenoxy propanol.

The remaining two compounds bear a structural resemblance to each other. Mucochloric acid is a simple compound with unusual structure having

halogen substitution, aldehyde, carboxyl group, and a double bond. The lactone of alpha bromobutyric acid would convert to an α-halogenated acid in case the ring were opened by an esterase.

In 1953, Fosdick and his co-workers[8] reported on a study of glycolysis inhibition among about 380 compounds, of which about 52 were halogen derivatives. Among his and our halogen derivatives there are only two compounds in common. One compound, 2-chloromethyl-5-hydroxy-1, 4-pyrone,

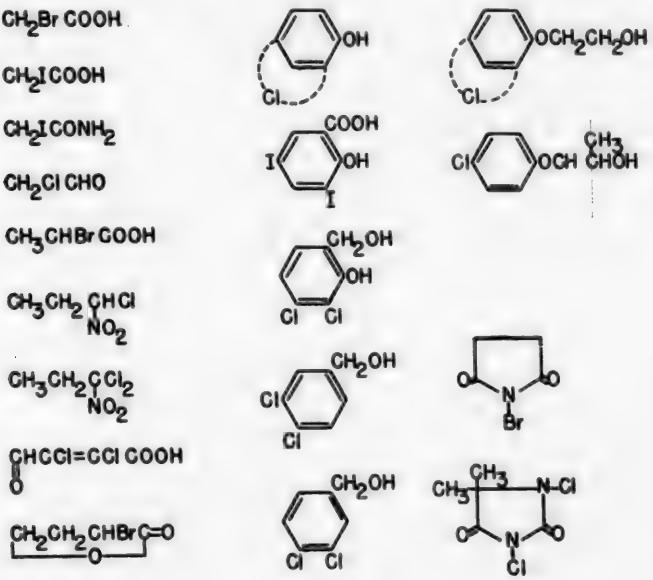

Fig. 1.

was inactive by both types of test. The other compound, 2, 2′-methylene bis (p-chlorophenol) was active at 0.005 per cent in Fosdick's tests, but not consistently inhibitory at saturation in 10 per cent propylene glycol in our experience. The substance is closely related to "hexachlorophene" (2, 2′-methylene bis [3, 4, 6-trichlorophenol]) which has been used as a germicide in soap, and found effective at 0.002 per cent or less in preventing glycolysis in pooled saliva.[9] This compound was also inactive by our method, possibly because of low solubility or restricted permeability through salivary sediment.[10]

SUMMARY

The influence of halogenated compounds on glycolysis by salivary sediment has been studied for nearly 500 compounds which were involved in a screening program. The purpose was to assess whether or not halogenation has any consistent influence on inhibitory action of a parent chemical. Among compounds in which either the parent structure or the monochloro derivative was active, halogenation had no consistent influence on the activity of the

parent structure, and sometimes produced large increases or decreases in inhibitory effect. Among derivatives both of which had slight or no activity, there was no difference in inhibitory action on the average, indicating no general trend induced by halogenation. The effect of multiple halogenation and the influence of fluorine, bromine, and iodine were likewise specific and unpredictable.

The inhibitory action of iodoacetate was marked and the specificity was high since iodoacetamide, bromoacetate, and bromopropionate were less inhibitory. Two halogen derivatives of nitropropane were inhibitory. Halogen derivatives of phenol, salicylic acid, benzyl alcohol, and saligenin were inhibitory. The related structures of phenoxy ethanol, phenoxy propanol, and and cyclohexylmethanol were active.

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CHEMICAL AND PHYSICAL STUDIES OF ENAMEL FROM HUMAN TEETH

IV. Density and Nitrogen Content of Mottled Enamel

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ON the basis of histologic studies (Black and McKay,[1] Williams,[2] and Ainsworth[3]), there is a general agreement regarding the developmentally defective nature of mottled enamel. Chemical analyses of the inorganic content by Armstrong and Brekhus,[4] Bowes and Murray,[5] Montelius and McIntosh,[6] Smith and Lantz,[7] and Ockerse[8] have shown no differences (ash basis) in the calcium, phosphorus, magnesium, and carbonate content of mottled and nonmottled teeth. The only study of the organic material by Bowes and Murray[9] demonstrated a higher nitrogen content in mottled enamel as compared to the nonmottled enamel.

It is obvious that the physical and chemical properties of mottled enamel have not been adequately studied. For instance, there is no information on the density of mottled enamel and only one study of its organic content. The purpose of this study, therefore, is to determine the density and nitrogen content of teeth showing different degrees of mottling in enamel.

MATERIAL AND METHODS

Mottled teeth for this study were obtained from Dr. S. Arnim of the University of Texas, Houston, Texas. The teeth showed varying degrees of mottling, ranging from mild to severe fluorosis (Figs. 1 and 2). Since the age of these teeth was not available, they could not be grouped according to their posteruptive age. The procedure for history recording, fixation and preparation of ground sections has been discussed in previous publications.[10, 11] The technic used for collection of mottled enamel samples was basically the same as the one formerly described.[12] Melon and Dallemagne's technic[11] for micropycnometric density and the Kjeldhal procedure for nitrogen determinations was used.

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Fig. 1. Fig. 2.

Figs. 1 and 2.—Photographs of teeth showing different degrees of mottling.

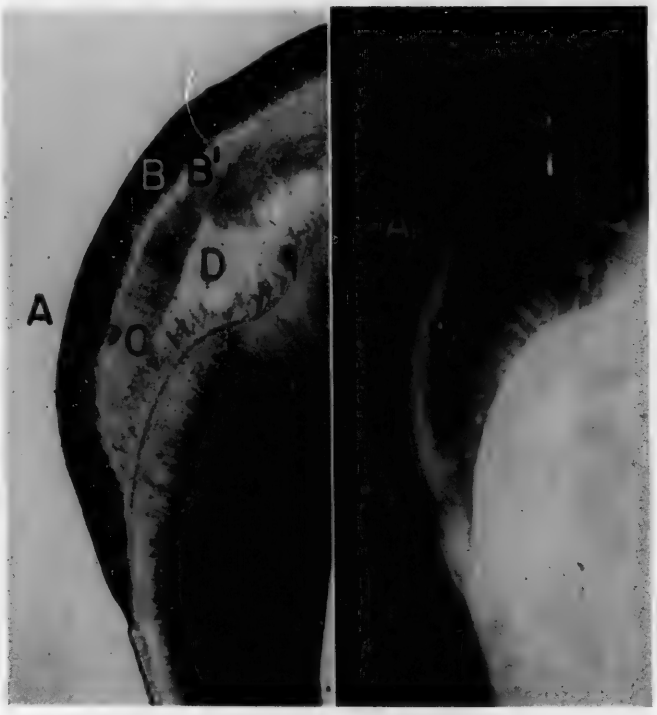

Fig. 3. Fig. 4.

Fig. 3.—Unstained ground section through a mottled enamel tooth. (Orig. mag. ×25.) *A*, Smooth surface contour. *B*, Pigmentation in the outer third of enamel. *B'*, Demarcation line between the pigmented and the nonpigmented enamel (*C*). *D*, Irregular translucent area.

Fig. 4.—Fluorophotomicrograph of the ground section through the mottled enamel tooth. (Orig. mag. ×25.) Note that the intensity of fluorescence follows a pattern similar to the pigmentation seen in the outer third of enamel.

RESULTS

Microscopic Study.—Ground sections from 16 mottled teeth were examined by transmitted light under low power (×100) of the microscope. The enamel in all sections showed a smooth surface contour, but the extension of the pigmented area into the enamel varied in every specimen.

The histologic structure shown in the following figures is representative of the observations on mottled enamel.

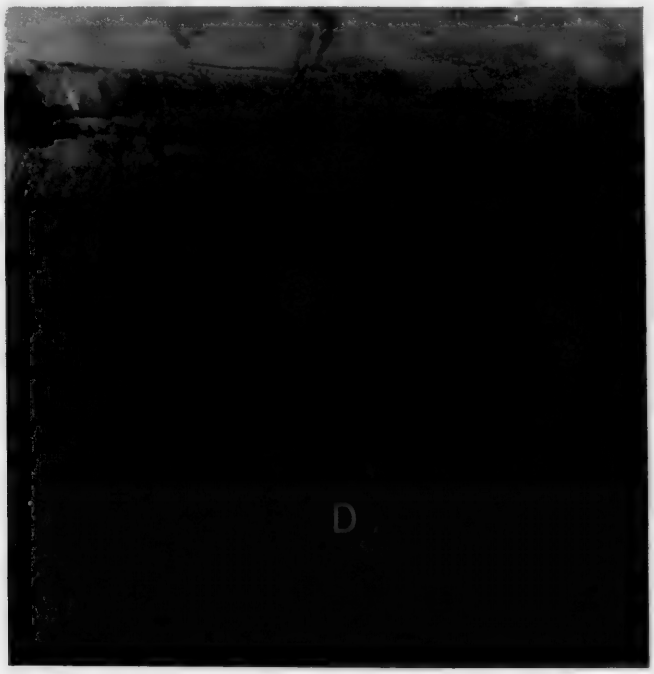

Fig. 5.—Unstained mottled enamel section (partially decalcified). (Orig. mag. × 210.) *A*, Surface membrane. *B*, Decalcified area showing the rod sheaths and cross striations of enamel. *C*, Optical artifact. *D*, Partially decalcified enamel.

Fig. 3 is an unstained ground section of a mottled tooth in which the smoothness of the enamel surface contour *(A)* is maintained. The outer third of the enamel *(B)* shows pigmentation which obscures the structure of the rod sheaths and incremental lines. The inner two thirds of the enamel *(C)* contains irregular translucent areas *(D)*. When examined under ultraviolet light (Fig. 4), the pigmented area in the outer third *(B)* of the enamel emits more intense fluorescence than the inner two thirds *(C)*. The line of demarcation *(B')* between the two regions appears dark in Fig. 4 but light in Fig. 3.

Fig. 5 represents a partially decalcified ground section of mottled enamel which had been exposed to the action of acid for about 72 hours. The surface membrane *(A)* is still intact. The structure of the rod sheaths and cross striations *(B)* in the outer third of enamel can be observed. The dark zone *(C)*

is an optical artifact, which always appears in advance of the active decalcification process. In the deeper layers *(D)* of the section, the structure of enamel rods and cross striations is visible.

Density and Nitrogen Determinations.—A comparison of the density and nitrogen determinations of mottled enamel and sound enamel is presented in Table I. It is evident that there is no difference in their densities, but the mottled enamel has a higher nitrogen content both on weight and volume bases.

It is interesting to note, when comparing the buccolingual and mesiodistal surfaces of mottled enamel (Table II), that the latter has a lower density and a higher nitrogen content.

TABLE I. COMPARISON OF MOTTLED ENAMEL AND SOUND ENAMEL

	DENSITY (GRAM/CM.^{3})			N_2 CONTENT (MG./GRAM)			N_2 CONTENT (MG./CM.^{3})		
	NO. OF TEETH	MEAN	S.E.*	NO. OF TEETH	MEAN	S.E.*	NO. OF TEETH	MEAN	S.E.*
Mottled enamel	161	2.88 ± .02		105	0.75 ± .05		105	2.17 ± .15	
Sound enamel	261	2.81 ± .02		282	0.56 ± .02		243	1.59 ± .06	

*The standard error is based on the within group standard deviations (pooled estimate), which are: Density, 0.29; Nitrogen (mg./gram), 0.57; Nitrogen, (mg./cm.^{3}), 1.54.

TABLE II. COMPARISON OF BUCCOLINGUAL AND MESIODISTAL SURFACES OF MOTTLED TEETH

Buccolingual surface	43	2.92 ± .04	38	0.63 ± .09	38	1.82 ± .25
Mesiodistal surface	45	2.82 ± .04	41	0.87 ± .09	41	2.47 ± .24

DISCUSSION

The findings on mottled enamel are in keeping with the chemical analyses of various investigators,[4-8] who found no difference (ash basis) in the calcium, phosphorus, and carbonate content of enamel from mottled and nonmottled teeth. Our findings on the increased organic content of mottled enamel are essentially the same as those of Murray and Bowes,[9] but the somewhat higher values reported by these authors are probably due to contamination of their samples with dentin.

Although this study was not directed toward determining the nature of the alteration in mottled enamel, some consideration should be given to this type of change. Black and McKay,[1] Williams,[2] and Ainsworth,[3] on the basis of their histologic studies, suggested that there was a deficiency of interprismatic substance which cause the defect in mottled enamel. It is possible that the pathways thus formed may permit diffusion of extraneous material to enter and produce pigmentation in the outer third of mottled enamel. The penetration of such extraneous material may also explain the increased nitrogen content in the mottled enamel.

SUMMARY

1. Mottled enamel has a higher organic content than sound enamel on the basis of nitrogen analyses.

2. There is no difference between the density of normal enamel and mottled enamel.

The author wishes to thank Dr. Sumter Arnim of the University of Texas, Houston, Texas, for the mottled teeth used in this study.

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PAROTIN AND GROWTH CENTERS OF FEMURS AND INCISORS IN MICE

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INTRODUCTION

EXTRACTS of salivary glands and saliva have been found to enhance mineralization and calcification of bones and teeth.[1, 2, 3] Concurrent with this activity, it has been observed that the blood serum calcium levels were lowered by approximately 15 per cent.[4, 5, 6] The substance responsible for these changes has been considered a "hormone-like" protein which has been prepared in purified and concentrated form from the parotid glands of young beef cattle and is known as "Parotin."[1, 7] A "parotin-like" substance has also been obtained from the urine of healthy young men and from human saliva.[8, 9] The use of radioactive P^{32} showed that when this "parotin-like" substance was administered to rats there followed a greater uptake of labeled inorganic phosphorus in the incisor teeth and femurs of these animals.[10]

In addition to the effect on mineralization, this substance had other effects among which was an activation of the adrenocortical function of the pituitary gland.[5] Recently, a hormonal relationship with regard to maintenance of the duct systems and histologic structure of the salivary glands has been established with the pituitary.[12, 13]

The interrelated and complex local events that take place during the mineralization and calcification of bones and teeth are not yet entirely understood.[14, 15, 16] Since salivary gland secretions and/or parotid gland extracts have effects upon mineralized tissues, it is of interest to determine possible histologic alterations they may induce in bones and teeth.

The continuously active areas in the growth centers of mice femurs and incisors are ideally suited for such a study since they may be altered by various bodily states or conditions.[17, 18]

This presentation constitutes a report on the histologic changes taking place in the epiphyseal discs of mice femurs and in the zones of enamel secretion of their mandibular incisors when a standardized and concentrated form of this "hormone-like" parotid gland extract was administered by intramuscular injections.

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METHODS

Young adult dba Rockland mice of similar weights and ages were used for these experiments. They were separately maintained as to sex.

The mice were injected periodically with the standardized preparation of the parotid glands obtained from young beef cattle and known as Parotin.*

A total of 120 males and 120 females, each weighing about 25 grams, received intramuscular injections of the hormone. In addition, 20 males and 20 females were carried as controls. The mice receiving the Parotin injections were divided into two groups. One group received a concentration of 0.15 mg. in normal saline and the other group received 0.30 mg. in normal saline at each injection. All injections were given on every third day. Animals were killed intermittently by chloroform inhalation, until a total of 10 injections were given. The mice were fed Purina Chow ad libitum.

Upon sacrifice, femurs and mandibles were immediately freed of adhering flesh and immersed in Zenker's solution where they remained for 12 hours. They were then washed in running tap water for 24 hours and afterward placed in 80 per cent alcohol for another 24 hours before decalcification.

Femurs and mandible halves were cut in midsagittal planes at ± 7μ. Staining was routine with hematoxylin and eosin.

For the present report, the epiphyseal plates or growth centers of femurs and the zone of enamel secretion of the mandibular incisors were studied.

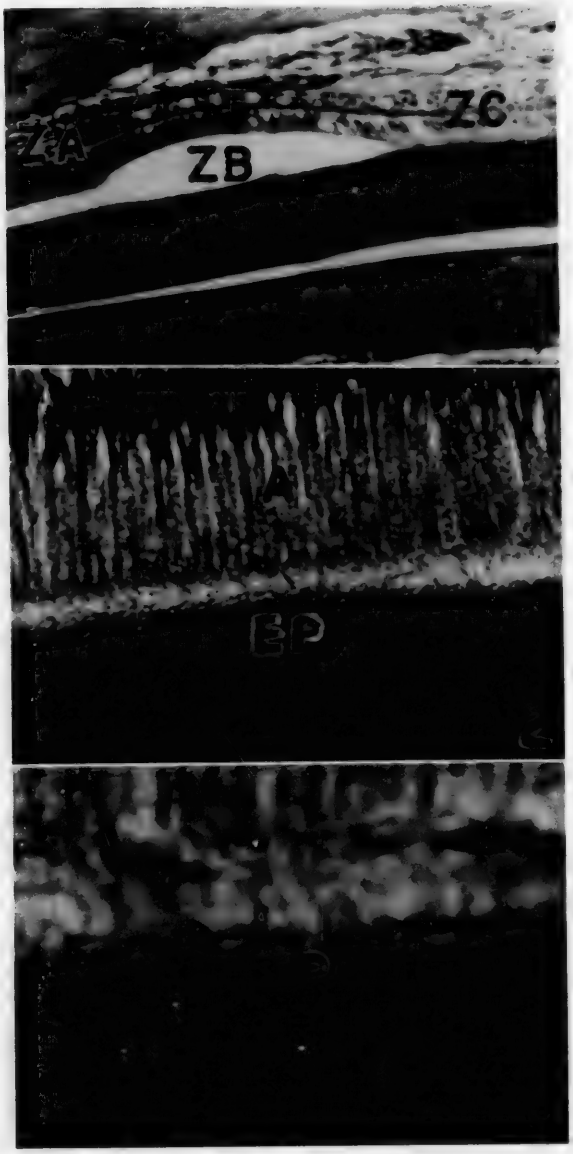
RESULTS

Growth centers and marrows of femurs and the enamel forming areas of mandibular incisors showed marked alterations following the administration of this salivary gland hormone preparation known as Parotin. These alterations became increasingly evident as the number of injections were increased. In addition, changes were more marked when Parotin was administered in 0.30 mg. per injection.

Disturbances in the ameloblasts in the secretory zone of the incisors became evident following 3 injections of the 0.15 mg. dose of Parotin. This is shown in Figs. 4 and 5 which are from a male mouse. A difference is readily seen by comparing these figures with Figs. 1, 2, and 3 which are from an untreated male mouse. By three injections of the salivary gland hormone, alterations were found in the ameloblasts, as well as in the forming enamel matrix. In Figs. 4 and 5 it is evident that the cytoplasm has become granular and that the walls of these cells have become more prominent than is usually found during secretory activity. The compartmental matrix formations have also lost much of their regularity. It is apparent that the matrix formation in Fig. 3, which represents a control or untreated animal, is quite different from the patterns seen in the incisors of treated animals.

After 7 injections of 0.30 mg. amounts, there was a severe distortion of the distal or secretory portion of the ameloblasts where the Tomes's processes deliver

*Manufactured by the Teikoku Hormone Manufacturing Company of Tokyo, Japan, and was supplied on request.



Key To Figures

A - Ameloblasts.	*RC* - Reverted cartilage cells.
BC - Ballooned cartilage cells.	*ZA* - Zone A.
BV - Blood vessels.	*ZB* - Zone B.
EP - Enamel protein.	*ZC* - Zone C.

Figs. 1 through 9 are from the enamel secreting area of Zone A of the mandibular incisors of dba Rockland young adult mice. In these figures ameloblasts are always to the top.

Fig. 1.—Enamel secreting area from mandibular incisor. (Orig. mag. ×285.)

Fig. 2.—Higher magnification of Fig. 1. (Orig. mag. ×1,273.)

Fig. 3.—Protein matrix from mandibular incisor. Higher magnification of Fig. 2. (Orig. mag. ×2,850.)

Fig. 4.—Ameloblasts in enamel secreting area from mandibular incisor of a male mouse. Three injections of 0.15 mg. of Parotin. (Orig. mag. ×285.)

Fig. 5.—Higher magnification of Fig. 4 to show ameloblasts and top of matrix in more detail. (Orig. mag. ×1,273.)

Fig. 6.—Enamel secreting area from mandibular incisor of a female mouse. Seven injections of 0.30 mg. of Parotin. (Orig. mag. ×283.)

Fig. 7.—Higher magnification of Fig. 6 to show ameloblasts and matrix in more detail. (Orig. mag. ×1,440.)

the protein matrix. Very little if any of the protein matrix is being secreted and with this extreme distortion, as seen in Figs. 6 and 7, which are from a female mouse, there is a complete loss of the pattern. In Figs. 8 and 9, which represent tissues from a male animal that received amounts of 9 injections of 0.15 mg., it is also apparent that the pattern is lost but not as severely as that shown in Figs. 6 and 7.

Fig. 8.

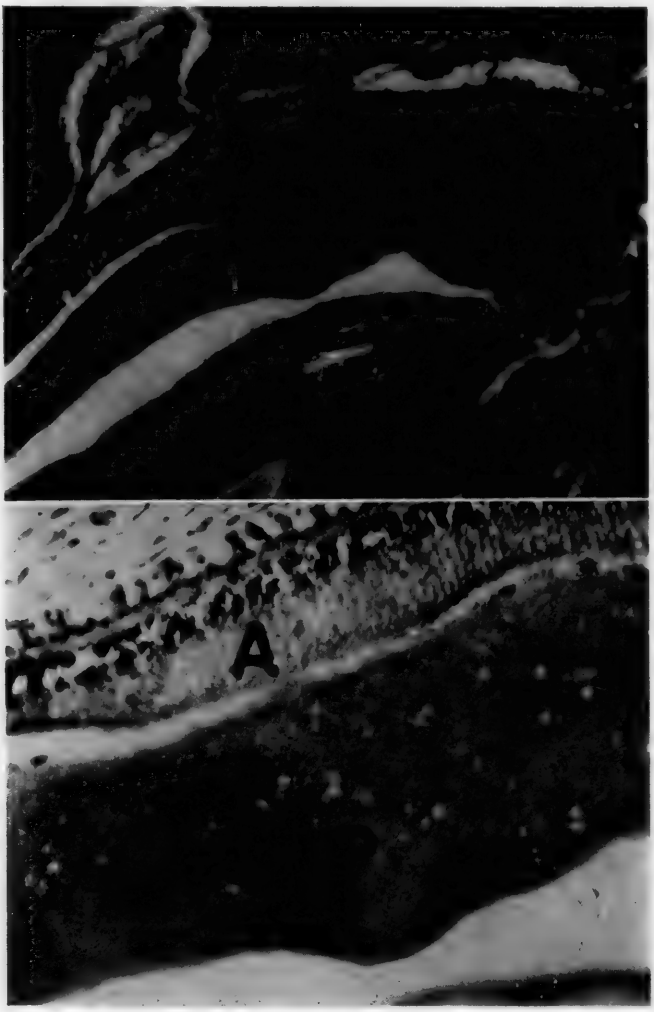


Fig. 9.

Fig. 8.—Enamel secreting area from mandibular incisor of a male mouse. Nine injections of 0.15 mg. of Parotin. (Orig. mag. ×113.)

Fig. 9.—Higher magnification of Fig. 8 to show alterations in matrix. (Orig. mag. ×1,275.)

It was also noted that there was considerable change in vascular beds of the incisors' growth centers, for blood vessels were considerably increased in size and number. There was also an increased number of red blood cells within them and this is demonstrated in Figs. 6 and 8.

Alterations taking place in the epiphyseal plates or growth centers of mice femurs were likewise marked when Parotin was administered. Figs. 10 and 11

Fig. 10.

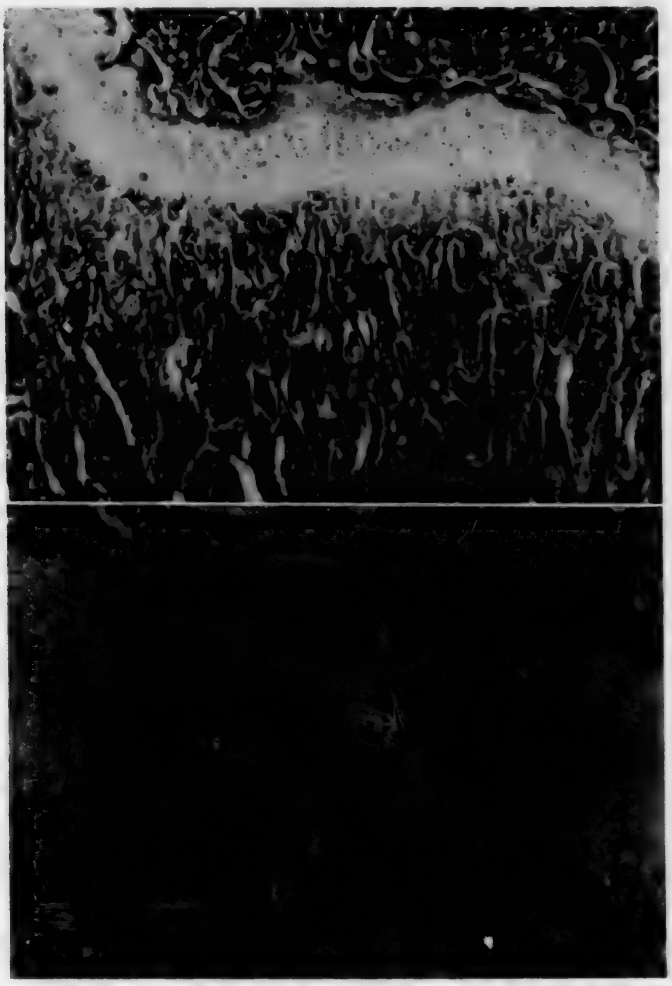


Fig. 11.

Figs. 10 through 17 are from the growth centers or epiphyseal discs of mice femurs. Terminal plates are to the top and trabeculae to the bottom.

Fig. 10.—Growth center or epiphyseal disc of femur from normal male mouse. (Orig. mag. ×93.)

Fig. 11.—Higher magnification of Fig. 10 to show cartilage cells. (Orig. mag. ×233.)

represent different magnifications of this area in the normal, untreated, male mouse femur. Early changes that took place following 3 injections of Parotin are shown in Figs. 12 and 13. The columns of cartilage cells as they approached the metaphyseal border did not degenerate as they did under normal conditions but underwent ballooning. This is shown in Fig. 13 and probably is an initial

Fig. 12.

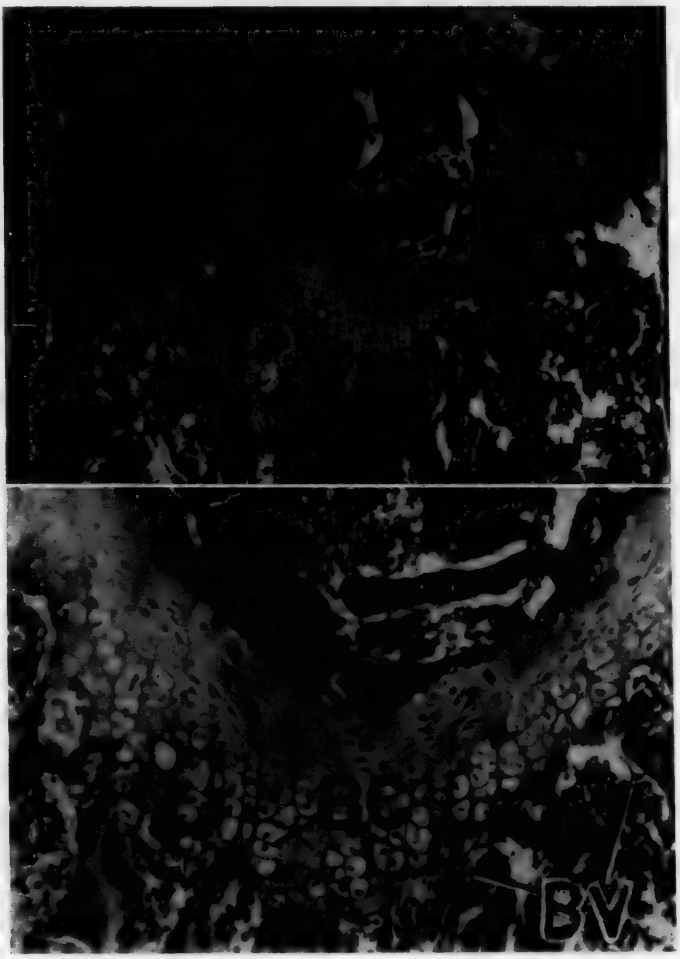


Fig. 13.

Fig. 12.—Growth center or epiphyseal disc of male mouse femur. Three injections of 0.30 mg. of Parotin. (Orig. mag. ×93.)

Fig. 13.—Higher magnification of Fig. 12 to show alterations at metaphyseal border. (Orig. mag. ×233.)

step toward the changes represented in Fig. 15. In addition, as compared with the controls shown in Figs. 10 and 11, there is a decrease in the proliferation of cartilage cells. This decrease was evident in all cases where Parotin had been administered.

Following the injections of Parotin, the number of blood vessels increased in the mice femurs along the metaphyseal border. The vessels were larger and were more fully packed with red blood cells. This increase in blood vessels continued so that, by 9 injections, the vessels along this border were very large

Fig. 14.

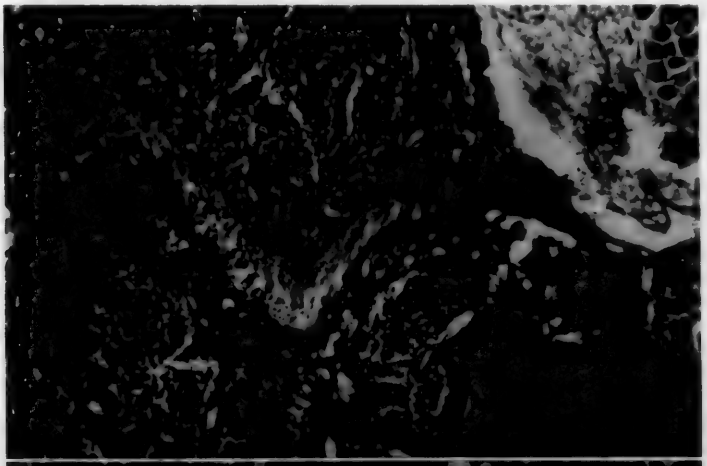


Fig. 15.

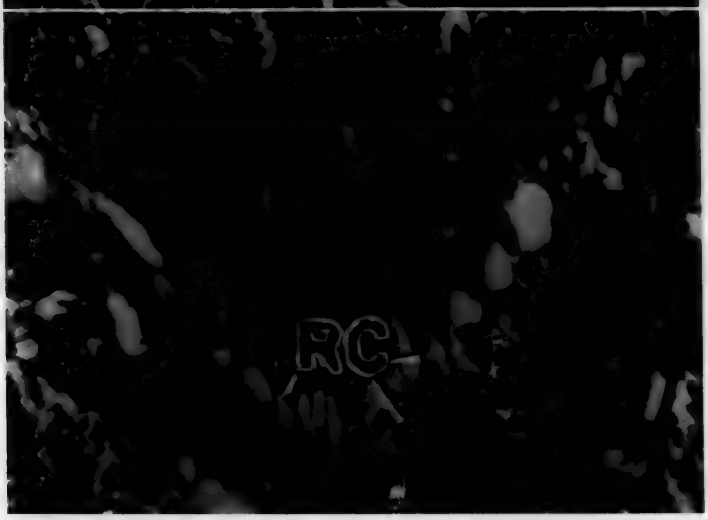


Fig. 14.—Growth center or epiphyseal disc of male mouse femur. Five injections of 0.30 mg. of Parotin. (Orig. mag. ×103.)

Fig. 15.—Higher magnification of Fig. 14 to show "reversion" of cartilage cells. (Orig. mag. ×233.)

and encroached directly against the epiphyseal cartilage plate. In addition, marrow was increased to the extent that the trabeculae were crowded out.

By 5 injections of the 0.30 mg. concentration per injection, there were further changes, particularly evident within the epiphyseal cartilage plates.

An example of this is shown in Figs. 14 and 15 which represent the epiphyseal plate in a male mouse. Here, there is almost a reversion of cartilage cells to connective tissue cells (if that is possible). The epiphyseal plate is not as thick as that seen in the previous instance (Figs. 12 and 13) and in the controls (Figs. 10 and 11). Blood vessels lying near the metaphyseal junction are not as numerous and not as large. However, there appears to be an encroachment

Fig. 16.

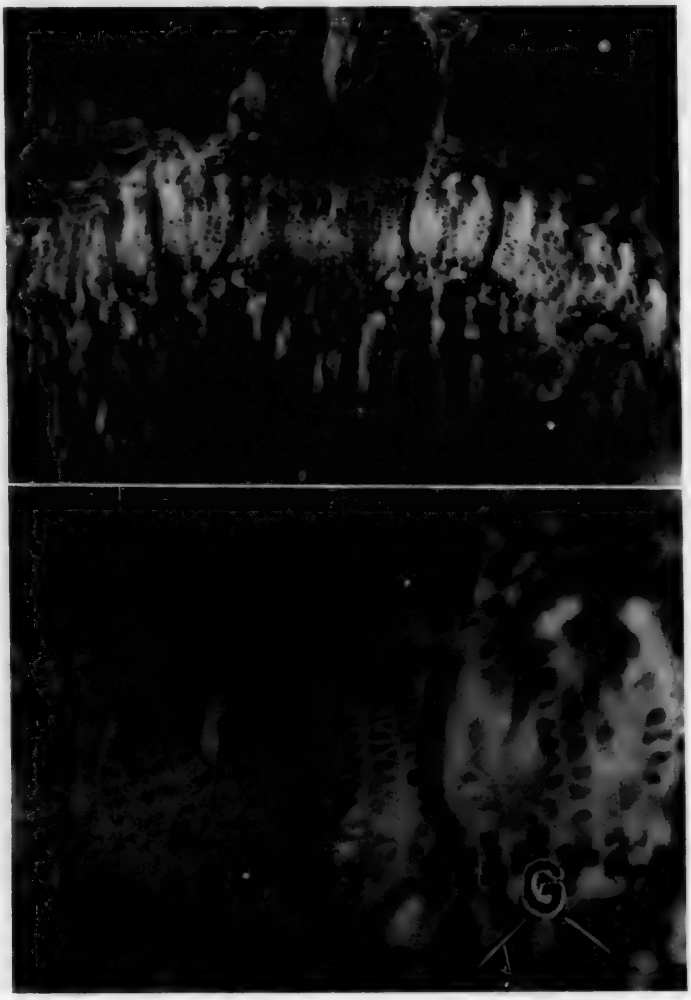


Fig. 17.

Fig. 16.—Growth center of epiphyseal disc of femur. Seven injections of 0.15 mg. of Parotin. (Orig. mag. ×113.)

Fig. 17.—Higher magnification of Fig. 16 to show encroachment of osteoid and failure of degeneration of cartilage cells. (Orig. mag. ×283.)

of marrow into this area and many multinucleated cells appearing like small megakaryocytes or giant cells are seen here. This is also emphasized in Figs. 16 and 17 where 7 injections of 0.15 mg. doses of Parotin had been given over a period of 21 days to a female mouse. In these latter instances, osteoid proliferations were found within the epiphyseal cartilage discs. The cellular components of the marrow appear to have been increased at the expense of the trabeculae which appear fewer and have less broad aspects. This increase in marrow is also apparent above the terminal plates on the proximal ends of the femur shafts. This is shown in Fig. 16.

After several injections, the male mice became vicious and difficult to handle. This was not true of the females. Gross examination showed the testes to be enlarged. Microscopically, there were increases in the germinal layers of the testes. Associated gonadal phenomena will be reported elsewhere. Male mice also became moribund more frequently than did the females.

DISCUSSION

These experiments have shown that the standardized preparation of the globulin-like protein extracted from salivary glands, known as "Parotin," has a negative effect on the growth centers of femurs and of mandibular incisors in Rockland mice. With the concentrations and number of injections used in the present experiments, mineralization and calcification were altered so that growth was not enhanced.

The concentrations used by Ito and Okabe[9] and by Ito, Aonuma, and Sawada[8] were stronger than those used in the present experiments. They used rabbits and obtained enhancement of growth in their experiments. In an experiment with rats where P^{32} was used, Ito, Tsurufuji, and Kubota[10] injected 2 mg. per 100 grams body weight and obtained an increased deposition of phosphorus in the growth areas of femurs and incisors which was presumably due to an increase in serum phosphorus levels as serum calcium levels were reduced.

While the above investigators did not usually continue treatment over the periods used in the present experiments, total amounts given were comparable to the amounts given in the present experiments where these marked changes became evident. In the present experiments, there were 1.2 mg. and 0.60 mg. used per 100 gram body weight of mice. With both of these concentrations, calcification was upset in the femurs and mandibular incisors. This was evident with both concentrations, by 3 injections over a period of 9 days.

It is interesting that Parotin has been found to have other effects on the blood in addition to altering the serum calcium and phosphorus levels.[8, 9] This has been manifested by an increase in the number of circulating leukocytes and a rise in bone marrow temperature.[9] Ito and Okabe claimed, however, that there was a continuing effect for 3 days on the serum calcium levels from a single injection of 2 to 3 mg. per kilogram body weight in rabbits.[2]

They, also, found that the rise in marrow temperature was likewise maintained for 3 days following a single injection of Parotin.[9] In the present instance, there was a marked increase in vascularity along the metaphyseal border of the epiphyseal discs and also in the vascular beds of the mandibular incisors. It is, therefore, possible that a rise in temperature resulted from the increased circulation of these areas of high metabolic activity. As manifested in the present experiments, the marked increase in size of the marrow spaces in the femurs retarded the degeneration and proliferation of chondrocytes so that the formation of trabeculae was interfered with. Perhaps the most important finding is the phenomenon associated with the apparent reversion of cartilage cells, a fact which in itself may shed more light on the interrelationship of mineralized tissues. In the vascular beds of the incisors, the increased blood supply may have been the indirect cause of the alteration on the productive activity of the secretory ameloblasts. This resulted in a complete upset of enamel formation.

Because a number of male animals became moribund and died by the time ten injections had been given, these experiments were not continued beyond the period of 30 days. While it was noted that these injections had a stimulatory effect on the germinal layer of the testes in male mice, phenomena associated with these events will be reported elsewhere. However, it does support a concept of the hormonal effect of the salivary gland extracts. The results suggest other lines of investigation and a possible correlation of salivary gland extracts and the activity of salivary glands with gonadal function as well as with the growth of mineralized tissues.

From these experiments it becomes necessary to undertake further investigations regarding the effect of this substance on the vascular beds in bodily regions of high metabolic activity. With regard to the bone marrow, increased numbers of megakaryocytes and/or giant cells also suggest avenues of future studies. It would, likewise, be interesting to relate osteoclastic activity to serum calcium and phosphorus levels as affected by Parotin. With regard to the secreting ameloblasts of Zone A*[4] in the mandibular incisors, it would be of importance to determine the extent of their recovery following administration of Parotin. The effect on dentin should also be considered.

SUMMARY AND CONCLUSIONS

Intramuscular injections of a parotid gland extract caused changes in the growth centers of mice femurs and in the zone of enamel secretion in the mandibular incisors. These changes were observed with injections of Parotin at 0.15 or 0.30 mg. for each injection. However, they were more extreme and took place sooner with the concentration of 0.30 mg. of Parotin per injection. Growth and development of these mineralized areas were not enhanced.

Changes in the enamel secreting zone of the mandibular incisors were as follows: (1) granular appearance of the cytoplasm, thickening of cell walls

*In a previous report on "Patterns of the Enamel Protein Elements of the Mandibular Incisors of Mice," this investigator found that there were three distinct layers which were designated as Zones A, B, and C. Zone A is the zone of secretion, Zone B is the zone of compression, not an artifact (see Fig. 1), and Zone C is the zone of reattachment of the ameloblasts.

of ameloblasts, (2) distortion of the ameloblastic Tomes's processes, (3) failure of ameloblasts to secrete compartmental protein arrangements in an orderly fashion, (4) distortion of the newly formed protein matrix, and (5) marked increase in vascularity of the area.

Changes that took place in the growth centers of the femurs were as follows: (1) retardation of expected degeneration of cartilage cells, (2) decrease in proliferation of cartilage cells, (3) ballooning and/or reversion of cartilage cells, (4) osteoid proliferation deep within the epiphyseal cartilage, (5) increased numbers of blood vessels along the metaphyses, and (6) increase in cellularity and size of marrow spaces.

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ELECTRON MICROSCOPE STUDIES OF THE SUBMICROSCOPIC STRUCTURE OF THE DENTINAL MATRIX

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DENTIN is generally referred to as a fibrillar calcified matrix permeated by dentinal tubules containing the process of odontoblasts.[1, 2] The dentinal tubules and the fibrils in the matrix have been the subject of histologic studies with the electron microscope,[3-6] but the calcified matrix has not been given the same attention. It has been described as a homogeneous calcified cementing substance[7] or a cement composed of inorganic salts and an organic material sensitive to the action of papain.[8] For this paper, the dentin matrix is defined as that region in dentin occupied by the mineral salts hydroxyapatite, and will include not only the intertubular region but also the outer calcified sheaths of the dentinal tubules.[3] Since prevailing histologic concepts of the fine structure of dentinal matrix are not clear, the present investigation was necessary to determine the microstructure of sheep's teeth as part of a programme being carried out to find the cause of the excessive wear when the sheep feed on certain improved pastures.[9] A comparison with human dentin was of interest and a sperm whale tooth was chosen to see if the largest mammal had identical dentin microstructure.

MATERIALS AND TECHNICS

The sheep teeth were central incisors of various ages and most were fixed in ethanol immediately after extraction. Some teeth, however, were fixed in osmic acid-Veronal buffer solution[10] and others were left unfixed and packed in ice until they could be prepared at the laboratory the following day.

The human teeth were premolars extracted for orthodontic reasons, and they were fixed in a similar manner to the sheep teeth. The sperm whale tooth, however, had been kept in a dry and unpreserved state for a period of two years. Most of the teeth were ground and polished with optical polishing powder, followed by fine powdered common salt in a saturated aqueous solution, to obtain flat dentin surfaces, and two-stage collodion carbon replicas were made of each surface, both before and after etching with selective reagents. Replicas were also made of the dentin surfaces of teeth which had been cleaved with a sharp chisel. After removing the collodion with amyl acetate, the carbon replicas were shadowed with uranium and then examined in a Metropolitan Vickers electron microscope, type EM2/1M.

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A number of reagents were used at room temperature to etch selectively the polished surface of human and sheep dentin and enable the organic and inorganic structure to be determined. These consisted of potassium hydroxide solutions at various strengths and sodium borate-boric acid buffer at pH 9 to hydrolyze the organic components; potassium hydrogen phthalate buffer at pH 4 and mineral acids at various strengths to etch both mineral salts and organic material; trichloracetic acid to remove the mineral salts and at the same time act as a protein precipitant; ethylene diamine tetraacetic acid as a chelating agent to remove the mineral salts; and the proteolytic enzyme trypsin to remove the organic material.

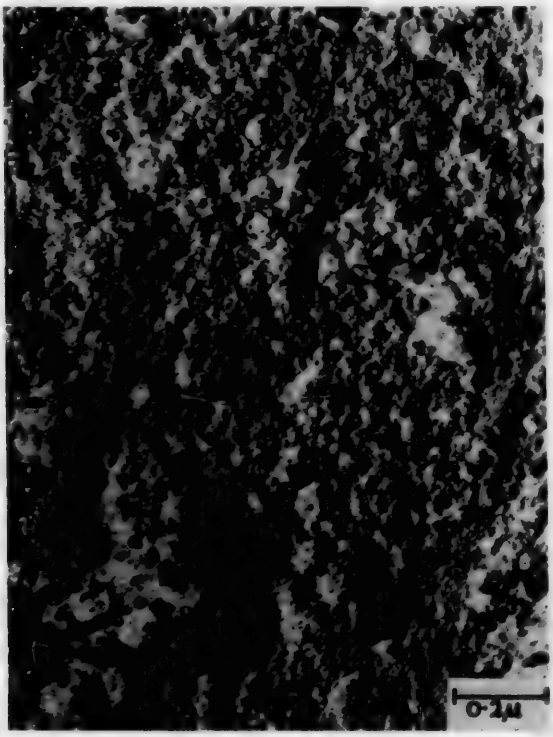

Fig. 1.—Surface of polished sheep's dentin after etching with potassium hydroxide at pH 10.4 for 4 hours.

The potassium hydroxide solutions were made from a 1 per cent solution and diluted until the desired pH was reached, e.g., 0.0035 per cent to give pH 10.4.

A carbon replica was prepared from each etched surface and compared with a replica of the same surface before etching. These micrographs were recorded at a magnification of ×5,000 and examined at a magnification of up to ×72,000. A total of more than 300 micrographs were recorded during these dentin studies.

RESULTS

The micrographs of mineral salts in the polished surfaces of osmic acid or alcohol-fixed dentin show a slightly granular structure when observed at magnifications of more than 50,000 diameters, but when these surfaces are etched with potassium hydroxide of pH 10.4 for 4 hours they then have the appearance of Fig. 1. The replica of many particles with a mean diameter of 320 Å can be

Fig. 2.—Pre-shadowed cleaved surface of human dentine showing distribution of fibrils in matrix.

seen and some spherical-shaped particles which became attached to the replica, and were opaque to the electron beam, are also evident. Dentin was etched at a faster rate when a stronger potassium hydroxide solution was used and at a slower rate when a sodium borate-boric acid buffer solution of pH 9 was used. The fact that the dentin matrix of both sheep and human teeth was etched in this manner by alkaline solutions suggested that the mineral salts were cemented together by an organic component which was hydrolyzed by such solutions, and

Fig. 3.—Polished surface of cementum in sperm whale's tooth.

evidence to this effect was obtained when the proteolytic enzyme trypsin etched a polished surface and outlined the 320 Å structure, although not as prominently as in Fig. 1.

An attempt was then made to dissolve the mineral salts in a polished dentin surface and observe the structure of this organic cementing component by using the chelating agent ethylene-diamine tetraacetic acid, but the results were inconclusive.

The use of mineral acids generally produced a relatively smooth dentin surface and it appeared that the organic components in the dentin were etched at much the same rate as the inorganic. Dilute acid etches produced some evidence of the 320 Å structure but not as significantly as the other reagents.

Carbon replicas were also prepared from the dentin surfaces of teeth which had been cleaved with a sharp chisel. Fig. 2 is a replica of dentin prepared by this method and shows the distribution of fibrils in the matrix between two odontoblast processes and demonstrates that the fibrils are not evenly spaced at a distance of the order 320 Å and, therefore, when hydrolyzed, are only partially responsible for the appearance of Fig. 1. The majority of fibrils in Fig. 2 are 200 Å diameter and can be identified by their hollow replicated ends. Some fibrils, however, have been torn from the dentin and transferred with the replica and these have a dark solid appearance.

When a replica of a cleaved surface was shadowed at a suitable angle and examined at magnifications above 50,000 diameters it then showed the same structure as Fig. 1. Since a cleaved surface is rough in comparison with one that is polished and etched, then only relatively small areas are shadowed at the best angle to reveal small structure.

Replicas of polished dentin and cementum of the sperm whale's tooth showed many 320 Å particles when examined at high magnification, as shown in Fig. 3.

DISCUSSION

The investigation of sheep's dentin was started with the knowledge that sheep's saliva is slightly alkaline during certain conditions and, since previous work had shown that 1 per cent solutions of potassium hydroxide had etched human dentin,[3] it was of interest to see if sheep's dentin was etched by weaker alkaline solutions.

Solutions of 0.0035 per cent at pH 10.4 and a buffer solution at pH 9 were used on both human and sheep's dentin and it was found that etching still occurred, although at a much reduced rate. It was thought at first that the mineral salts were released when the fibrils permeating the dentin[1, 2, 8, 12] were hydrolyzed, but it was impossible to see how an alkaline-etched surface, such as Fig. 1, could be produced by hydrolyzing the fine fibrils which intercept the dentin surface as shown in Fig. 2. Adjacent fibrils are spaced at distances ranging from 200 Å upward and their distribution is too irregular to separate the mineral salts into particles as small as 320 Å. This suggested that an additional organic component was present which cemented the minute particles of dentin and this theory was supported when a dentin surface was etched with trypsin and the same fine structure revealed.

Attempts to isolate this organic cementing material with a chelating agent have met with little success and this is not surprising since initially, such material will have very poor contrast in the electron microscope and, second, a resolving power of better than 50 Å will be essential.

When we consider the micrograph of the polished sperm whale's tooth (see Fig. 3), we find that the surface contains an abundance of loose particles roughly spherical in shape. The fact that the particles have been seen on a polished dentin surface in such profusion, only on a tooth which had been kept

in a dry state for some years, suggests that the cementing medium has deteriorated. It can also be seen that the particle size of the mineral salts in dentin and cementum in a large mammal, such as the sperm whale, is the same as that in human or sheep dentin.

It was observed by Rouiller, Huber, and Rutishauser[8] that the matrix of human dentin was etched by the proteolytic enzyme papain but, since magnifications above ×20,000 were not used, the ultrafine structure was not apparent. The presence of human dentin crystallites with a size of about 300 Å have, however, been measured by Trautz[11] and Thewlis[13] by means of x-ray diffraction.

CONCLUSIONS

The present investigations on fixed and unfixed teeth have disclosed that the mineral salts in the dentin matrix of sheep, human, or sperm whale teeth are roughly spherical crystallites with a mean diameter of 320 Å and are cemented by an organic component which is etched by acid solutions of pH 4, by trypsin, and by alkaline solutions at least as weak as pH 9.

The author is indebted to Mr. J. A. Garrick, Zoology Department, Victoria University of Wellington, for the sperm whale tooth, and to Mr. L. M. Adamson of Dominion Physical Laboratory for the photographic processing.

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REMOVAL OF RADIOACTIVE FAT AND PROTEIN FROM THE MOUTH

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RADIOACTIVE iodine labeled test meals are widely used for the diagnosis of malabsorption states such as sprue and pancreatitis.[1, 2] The observation that detectable amounts of tracer material remained in the mouth after swallowing the tagged test meal prompted this investigation to determine the amount of material that remained after a simulated meal, and the means of clearing residual particles from the oral cavity.

The usual procedure for determining the clearance of food particles from the oral cavity involves the chewing of a test cracker followed by measurement of residual debris in collected saliva and successive mouth rinsings.[3] Some useful information about the relative effectiveness of various cleansing and rinsing procedures has been obtained by this method; however, to date there has been no accurate means of testing for complete clearance of food particles from the mouth. Through the use of radioactive isotopes, a test reflecting the actual conditions in the mouth before and after cleansing has been developed.

METHOD

Two microcuries of radioiodinated human serum albumin or triolein* were mixed thoroughly with peanut butter in a Waring Blendor for 15 minutes and spread on soda crackers. Four healthy medical students and one of the authors (F. K. B.) served as test subjects. Their thyroid glands were blocked with 10 drops of Lugol's solution on the day of the experiment and for several days later to prevent uptake of radioactive iodine from the metabolized test substances.

A wide angle scintillation counter with a thallium activated sodium iodide crystal, connected to a scaler, was used as a radiation detector. The tube was pointed toward the oral cavity through the cheek of the test subject (Fig. 1). The test subject then put a soda cracker covered with the radioactive material in peanut butter in his mouth and the radioactivity of this test meal was measured. The background of the instrument was subtracted from the counts of radioactivity obtained and the net counts were expressed as 100 per cent since

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the entire test meal was measured. Forty-eight technically satisfactory experiments were carried out; 12 with radioactive albumin and 36 with radioactive triolein.

The test subject chewed and swallowed the cracker and peanut butter. The entire experiment was designed to compare with an everyday situation and an effort was made to chew and swallow as normally as possible. The residual radioactivity was then measured. The test subject rinsed his mouth with either cold tap water or hot coffee, again in an attempt to duplicate a normal American meal. The remaining radioactivity was measured again.

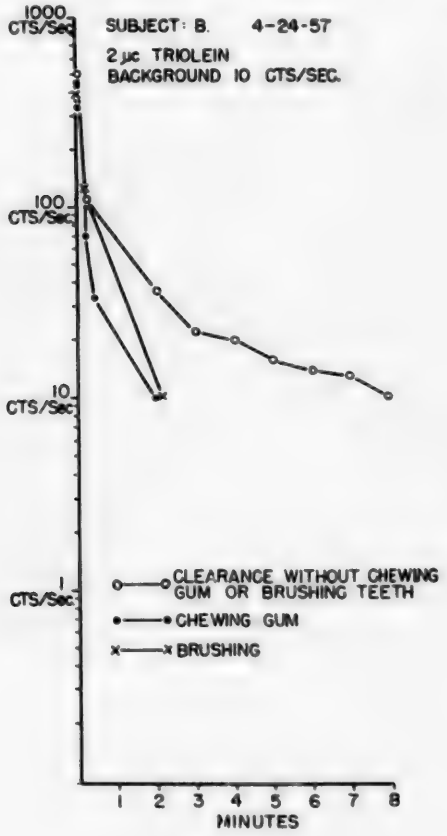

Fig. 1.

Three different procedures were undertaken. (1) Counts of radioactivity were taken at 1- or 2-minute intervals until background was reached. Each experiment was terminated after 10 minutes. Following rinsing, the subjects were asked either (2) to chew one stick of chewing gum, or (3) to brush their teeth in a normal fashion for 1 minute, using toothpaste. Counts of radioactivity were taken at 1- or 2-minute intervals until background was reached.

RESULTS

Substantial amounts of radioactive material remained in the oral cavity after chewing and swallowing the test cracker and peanut butter. The quantities ranged from 4 per cent to 33 per cent of the administered dose with a mean of 18 per cent for the 48 experiments conducted.

Rinsing the mouth with water or hot coffee reduced the radioactivity to between one third and one half the amount remaining after chewing and swallowing. (In eight experiments, during which counts of radioactivity were taken at 1- or 2-minute intervals after rinsing, it was found that the background level of radioactivity was reached in 5 to 10 minutes.) In two experiments the mouth was clear after rinsing.

Fig. 2.

Each of the 5 test subjects chewed one stick of gum for 1 minute after the rinsing procedure. In 1 case the mouth was clear in 5 minutes. In all other cases the mouth was free of radioactive material at the end of 1 minute of chewing.

Rinsing and brushing the teeth for 1 minute reduced radioactivity to the background count within 3 minutes in all test subjects. Since brushing could not be done near the instruments because of possible contamination, it took a minute to re-seat the subject in front of the scintillation tube, thus accounting for the slightly longer interval of time before the last count in this experiment.

Fig. 1 illustrates the typical rate of clearing of radioactive material from the oral cavity of Subject B using the three methods described. Fig. 2 shows the scintillation tube in place.

CONCLUSIONS

A new method for assessing food clearance from the mouth, utilizing radioiodinated triolein and human serum albumin blended with peanut butter, was tested in 48 experiments. There was no difference in the rate of removal of the two compounds. After chewing and swallowing the respective radioactive compounds, an average of 18 per cent of the radioactive material remained in the mouth. The residual radioactive fat and protein was further reduced by (1) rinsing with water or hot coffee, (2) chewing of gum, and (3) tooth brushing. Chewing gum and the use of the toothbrush were equally effective, attaining a greater reduction than the rinsing.

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ORAL CHANGES IN NORMAL AND SCORBUTIC GUINEA PIGS INJECTED WITH ASCORBIC ACID-1-C^{14}

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INTRODUCTION

THE USE of various vitamins in the treatment of some oral pathologic conditions has been recognized. One of the vitamins that has long held the interest of the dental profession is ascorbic acid since scurvy and some forms of gingivitis have been known to respond to the oral administration of the vitamin. It should be borne in mind that this vitamin is used in practice in spite of the fact that our knowledge of its role in the metabolism of oral tissues is not understood.

In scientific investigations to determine the function of ascorbic acid in oral tissues, it has been customary to utilize the research technics of a single basic science. However, in combining the efforts of the separate research disciplines in radiology, histology, and biochemistry, as we have attempted in our study, a novel, more comprehensive approach has been provided for the analysis of the basic mechanisms in ascorbic acid metabolism by oral tissues.

In our studies utilizing normal and scorbutic guinea pigs injected intraperitoneally with radioactive ascorbic acid, radioautographic methods localized radioactive uptake in pulp, periodontal membrane, alveolar periosteum, cortical and supporting bone. By histochemical staining technics, the vitamin was identified and localized adjacent to the odontoblastic layer of the pulp, in the interlobar and interlobular connective tissue of the submaxillary gland and within the adrenals. By biochemical analysis, we were able to correlate the radioautographic and histochemical findings by identifying the same pattern of distribution of radioactivity in the tissues described. Neither radioautography, histochemical staining for ascorbic acid, nor chemical analysis revealed any ascorbic acid in the enamel or dentin. In addition, our biochemical studies revealed that these oral tissues showing specific pathology

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(pulp, periodontal membrane, cortical and supporting bone) had the highest concentration of radioactive, non-ascorbic compounds. The liver, although of course requiring the vitamin, did not show as profound a pathologic change, and had the lowest concentration of these radioactive, non-ascorbic acid compounds. The findings in the oral tissues may be a reflection of physiologic utilization of ascorbic acid to repair the damage occurring during the scorbutic period.

From our chemical data, we concur with other reports in the literature that the amount of radioactive ascorbic acid incorporated into the various tissues is approximately the same in the scorbutic as in the normal animal. We found, however, upon further examination of our radioautographs that, on a tissue level, there is a difference in the pattern of distribution of radioactivity within specific tissues when the normal and scorbutic jaws are compared. The heavy uptake of radioactivity in the mandibular cortex of the normal animal suggested the possibility that this finding was an expression of the growth function in the normal. Little uptake in the mandibular cortex of the scorbutic animals, coupled with their marked weight loss, seemed to indicate that the growth process was markedly slowed. The process of active repair was evidenced in the scorbutic animals in heavy uptake of radioactivity in epiphytic bone and in the bone-embedded fibers and cementum-embedded fibers of the periodontal membrane.

MATERIALS AND METHODS

Two series of studies were completed; one with 76 animals sacrificed 2 hours to 96 hours after the injection of the labeled vitamin, while in the other series, 18 animals were studied in the 5 to 60 minutes time interval. This report is concerned with the short-time interval series.

All animals were fed an ascorbic acid free diet. The control animals received a daily oral supplement of 6 mg. of ascorbic acid and were permitted to have only as much food as the experimental animals. After a period of 3 weeks, clinical symptoms of scurvy became apparent in the experimental animals. During the diet control period, the control animals gained an average of 92 grams, the two saturated animals (50 mg. daily supplement) gained 101 grams, while the experimental group lost an average of 57 grams. Each animal was injected intraperitoneally with 1.5 mg. of ascorbic acid-1-C^{14} of 11.1 μc activity immediately before sacrifice. The animals were sacrificed by decapitation.

	5 min.	10 min.	15 min.	60 min.
Experimental animals	2	2	2	2
Pair-fed controls	2	2	2	2
Saturated animals	1		1	

Liver, and the adrenal and submaxillary glands were removed and stored frozen under a nitrogen atmosphere until chemically analyzed. One half of

the mandible was used for radioautographic study; the other half was reserved for histologic or biochemical study. The maxilla was further dissected to yield additional incisal and molar pulp, as well as enamel and dentin for biochemical studies.

Radioautographic Methods.—

After dissection, one half of each mandible was frozen immediately in alcohol and dry ice. In preparation of ground sections, the jaws were individually embedded in dental impression plaster. The jaws were sectioned using the Hauptfuehrer-Spongberg apparatus[1] and maintained in a frozen state using liquid nitrogen. Mineral seal oil was used as a coolant during the sectioning procedure to prevent redistribution of the water soluble radioactive material. Sections were made transversely through both incisor and molar

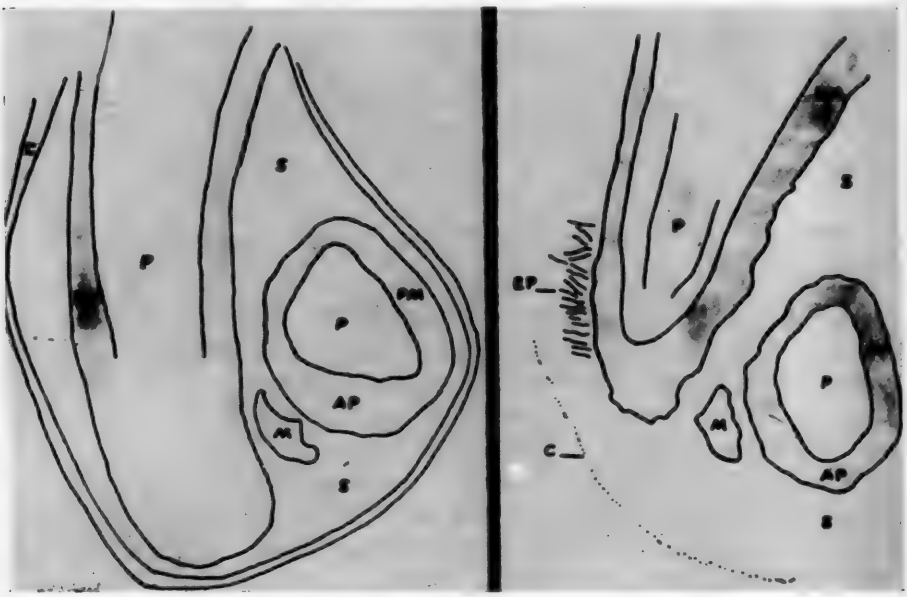

Fig. 1.—*A*, Normal animal and *B*, scorbutic animal (× 13).
Semidiagrams of radioautographs of frontal sections through the mesial roots of the mandibular first molars of guinea pigs sacrificed 60 minutes after injection of radioactive ascorbic acid. *P*—pulp; *PM*—periodontal membrane; *AP*—alveolar periosteum; *C*—cortical bone; *S*—supporting bone; *M*—mandibular canal; and *EP*—epiphytic bone.

areas. Sections were cut at 200 μ in the normal animals. Because of the fragility of the jaw specimens of the scorbutic animals, these sections were cut at 375 μ.

The method of Wainwright[2] was employed in making the radioautographs. In the darkroom, a piece of Kodak Ultra-Speed dental x-ray film was placed upon each side of the specimen and sandwiched between two 1 by 3 inch glass slides. A clamp was placed over the unit to ensure direct and constant contact between emulsions and specimen. The emulsions were exposed for 30 days in a constant temperature-humidity chamber maintained at a constant

temperature of 50° F. and at a relative humidity of 50 per cent. At the termination of the exposure period, each film was processed in rapid medical x-ray developer for exactly 5 minutes and subsequently fixed for 20 minutes.

Radioautographic Findings.—

A comprehensive examination of the radioautographs encompasses an assessment and comparison of the radioautographs of normal and scorbutic jaw specimens from the 60-minute through the 5-minute survival groups. Figs. 1 and 2 are semidiagrammatic examples of the differences in the pattern of radioactive uptake between the normal and scorbutic animals.

Fig. 1 represents radioautographs of frontal sections through the mesial root of mandibular first molars of a normal and a scorbutic guinea pig. In the radioautograph of the normal guinea pig jaw, evidence of radioactive

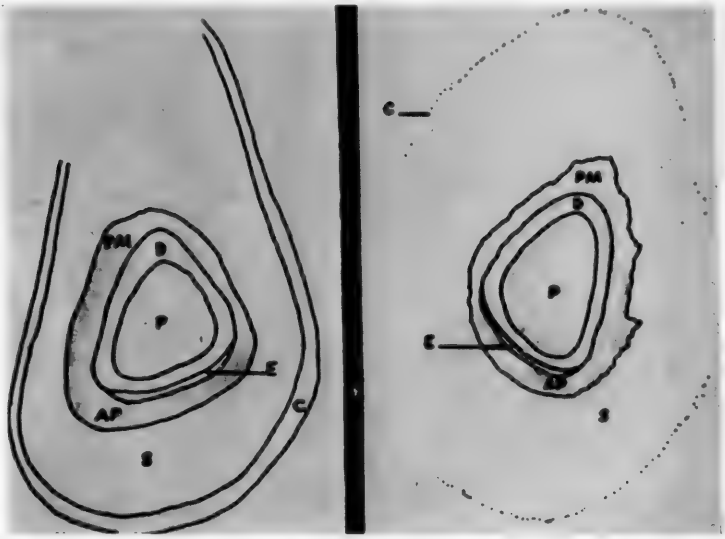

Fig. 2.—*A*, Normal animal and *B*, scorbutic animal (× 13).

Semidiagrams of radioautographs of cross section through the mandibular incisors, just anterior to the first molars, of guinea pigs sacrificed 60 minutes after injection of radioactive ascorbic acid. *P*—pulp; *PM*—periodontal membrane; *AP*—alveolar periosteum; *C*—cortical bone; *S*—supporting bone; *D*—dentin; and *E*—enamel area.

uptake is seen in pulp *(P)*, periodontal membrane *(PM)*, alveolar periosteum *(AP)*, cortical bone *(C)*, supporting bone *(S)*, and mandibular canal *(M)*. The radioautograph of the scorbutic jaw shows, in addition, epiphytic bone *(EP)*.

Fig. 2 shows radioautographs of cross sections through mandibular incisors, just anterior to the first molar, of a normal and scorbutic guinea pig. With the exception of epiphytic bone, all structures described in Fig. 1 are seen. In addition, Fig. 2 demonstrates tissues showing no uptake of radioactivity—dentin *(D)* and enamel area *(E)*. A description of the radioactive uptake and respective differences in distribution as seen in radioautographs of normal and scorbutic jaws beginning with the 60-minute survival groups follows.

Sixty-minute survival groups: In the normal group, Fig. 3 is representative of the distribution of radioactive uptake. The lamellated bone of the mandibular cortex is well defined in a regular outline. As viewed in the molar sections, the masseteric ridge is prominent, but there is no evidence of radioactivity in the adjacent supporting bone. However, more dense irregular islands of radioactive uptake appear in the supporting bone near the medial crest of the molar alveolus and in the basal bone inferior to the mandibular canal. The medio-superior border of the mandibular canal is well defined and of a density comparable to cortical bone. A homogeneous distribution of

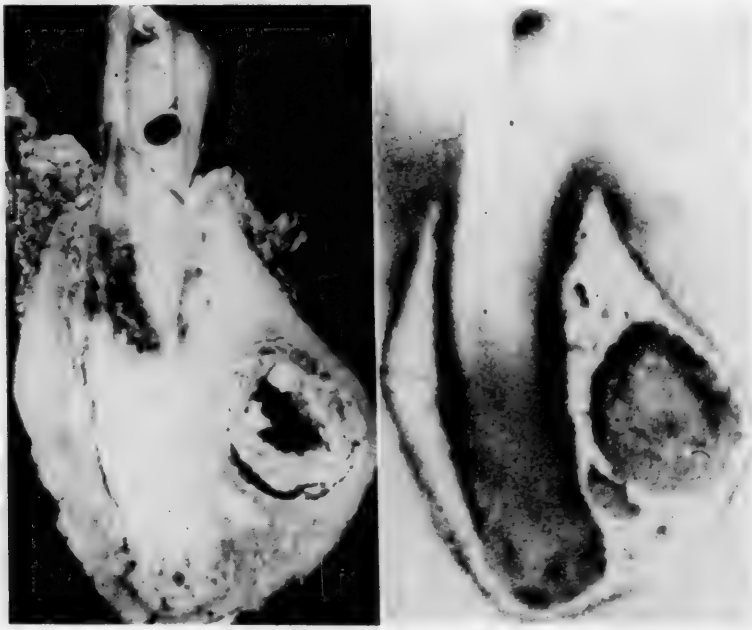

Fig. 3.—Frontal section and companion radioautograph. Section cut through the distal aspect of the mesial root of the mandibular first molar of a normal guinea pig (No. 208) sacrificed one hour after injection of radioactive ascorbic acid (× 15). Radioautographic exposure on Kodak Ultra-Speed dental x-ray film after 30-day contact.

a slightly lesser density is seen in the lumen of the canal. At the level of the normal incisor (Fig. 4), just medial to the first molar, the distribution of radioactive uptake in cortical bone is again well defined. However, there is a wider band of density in the inferior lateral portions than in either the medial or superior aspect. Small, irregular, dense islands of immature bone are visible medially and inferiorly to the cross section of the incisor tooth.

In Fig. 5, the scorbutic mandibular cortex is poorly defined. A wide distribution of density is seen in supporting bone. Whereas the section from the normal showed scattered uptake of radioactivity, the scorbutic pattern of radioactive uptake in the molar section appears as dense lines of epiphytic bone radiating toward the lateral border of the jaw, and small, irregular islands of density located in the supporting bone along the lower border of

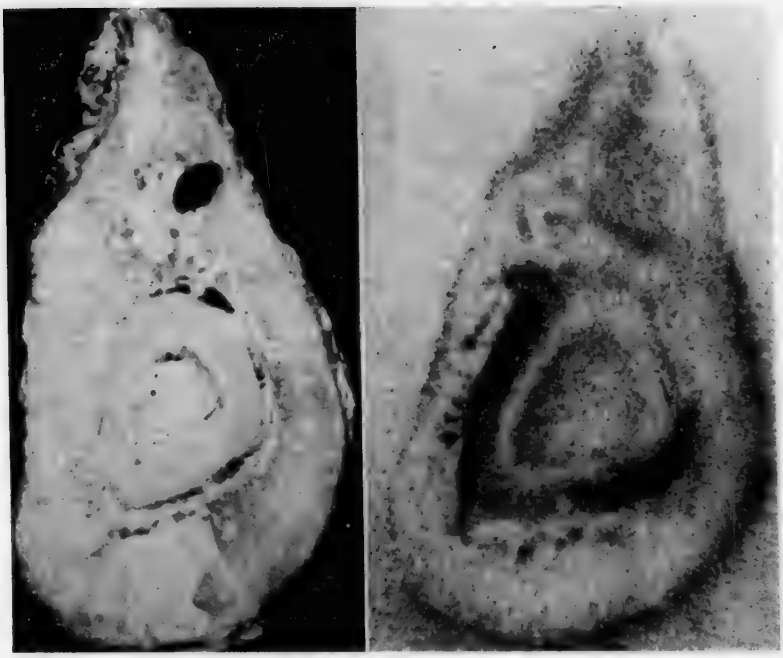

Fig. 4.—Cross section and companion radioautograph. Section cut through mandibular incisor, just anterior to the first molar of a normal guinea pig (No. 208) sacrificed one hour after injection of radioactive ascorbic acid (× 15). Radioautographic exposure on Kodak Ultra-Speed dental x-ray film after 30-day contact.

Fig. 5.—Frontal section and companion radioautograph. Section cut through the center of the mesial root of the mandibular first molar of a scorbutic guinea pig (No. 204) sacrificed one hour after injection of radioactive ascorbic acid (× 15). Radioautographic exposure on Kodak Ultra-Speed dental x-ray film after 30-day contact.

the mandible. Larger islands of radioactive uptake appear more widely scattered throughout the supporting bone medial to the molar alveolus. The mandibular canal is seen as a dense mass. With the exception of an area of very heavy concentration of radioactive uptake opposite and just below the masseteric ridge, the periodontal membrane of the molar is of a homogeneous density throughout in the normal animal (Fig. 3). There is no evidence of a division of this tissue into the three areas of cementum-embedded fibers, bone-embedded fibers, and fibers of the intermediate plexus. A similar pattern of radioactive uptake is seen in the periodontal membrane near the basal end of the incisor.

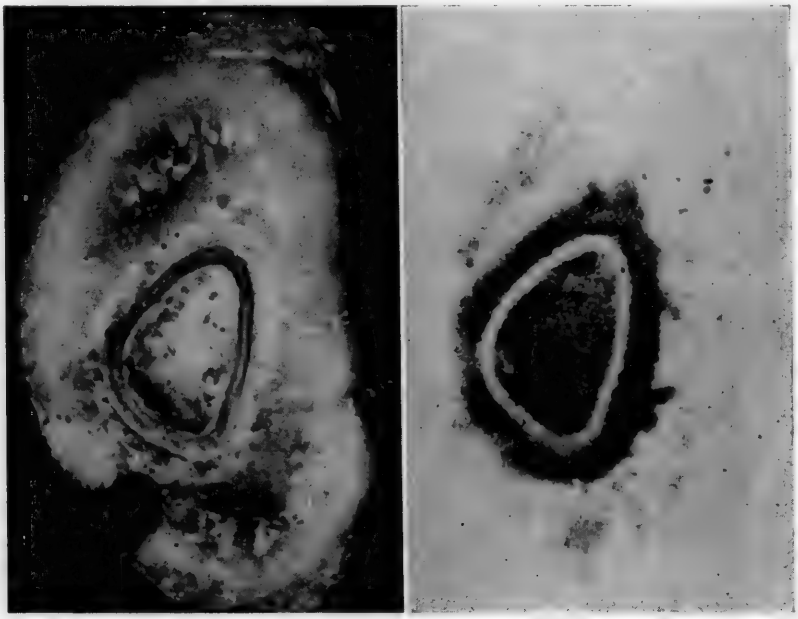

Fig. 6.—Cross section and companion radioautograph. Section cut through the mandibular incisor, just anterior to the first molar of a scorbutic guinea pig (No. 203) sacrificed one hour after injection of radioactive ascorbic acid (× 15). Radioautographic exposure on Kodak Ultra-Speed dental x-ray film after 30-day contact.

In the normal incisor sections (Fig. 4), there is evidence of diffuse penetration throughout the periodontal membrane and alveolar periosteum. The heaviest concentration occurs in the medial aspect of the periodontal membrane and in the lateral inferior portion of the alveolar periosteum. The lateral superior region of the periodontal membrane is thin, less dense, and somewhat irregular in outline. The borders of both periodontal membrane and alveolar periosteum are relatively smooth, and both of these tissues demonstrate the heaviest uptake of radioactivity of all tissues examined.

When viewed in the radioautographs of the root section of the scorbutic incisor (Fig. 6), the periodontal membrane and alveolar periosteum (as in the normal tissue) appear as the areas of heaviest concentration of radioactivity.

The border of the cementum-embedded fibers of the periodontal membrane is smooth. However, the area of bone-embedded fibers is irregular and, at intervals, projects well into the supporting bone. In the scorbutic molar periodontal membrane (Fig. 5), small areas of increased density are interspersed throughout this tissue. In addition, there is a suggestion of organization of these areas into bands bridging cementum and alveolar bone proper. Cross sections through the incisor tooth (Fig. 6) demonstrate three distinct periodontal areas in contrast to a homogeneous distribution in the normal. The cementum-embedded fibers and the bone-embedded fibers are seen as narrow, dense bands. The fibers of the intermediate plexus are evidenced as a broader, less dense band.

In the normal guinea pig (Fig. 3), pulpal uptake of radioactivity in the molar is evenly distributed and of a density comparable to alveolar periosteum and mandibular canal. Diffuse distribution of radioactivity is seen in the pulp of the incisor section with heaviest concentration at its periphery.

The pulp of the scorbutic animal (Fig. 6) shows a homogeneous uptake of radioactivity of a density greater than that of normal pulp.

Fifteen-minute survival groups: The radioautographs of both normal and scorbutic jaws of these groups are somewhat less dense than those of the corresponding 60-minute survival groups.

Again, in the normal jaws, radioactive uptake in the cortical bone of the mandible is evidenced by a dense but, somewhat, diffuse band. The width and density of this band of cortical bone is increased on the medial aspect of the mandible as viewed in the molar sections. Dense, irregular islands of radioactivity are seen in the supporting bone of the incisor alveolus. The most superior portion of supporting bone in the incisor sections is seen as an area of diffuse penetration. In the mandibular canal, the radioactive uptake is homogeneous and of a density comparable to the diffuse areas in supporting bone.

In the scorbutic group, the general pattern of distribution of radioactivity closely follows that seen in the scorbutic animals sacrificed 60 minutes after injection. Again, mandibular cortex appears poorly defined and a wide distribution of radioactivity is evident in supporting bone. Dense lines of epiphytic bone radiate outward from the molar alveoli. Islands of density are scattered throughout the supporting bone. The mandibular canal is seen as a less dense mass in the sections through the basal end of the incisor.

In the molar sections of the normal animals, the periodontal membrane is of a heavy, but homogeneous density throughout. Both cementum and bone borders are relatively smooth. In both periodontal membrane and alveolar periosteum, as viewed in the incisor sections, the uptake of radioactivity is heterogeneous.

In the scorbutic group, both periodontal membrane and alveolar periosteum show the greatest relative uptake of radioactivity. As in the 60-minute survival scorbutic group, the border of cementum-embedded fibers is smooth,

while the area of bone-embedded fibers is irregular. In the 15-minute sacrifice group, the more dense bands bridging cementum and alveolar bone proper are seen less frequently than in the 60-minute group. In the cross section through the incisor, there is a suggestion of a division of the periodontal membrane into three zones only in certain areas.

The basal end of the incisors in the normal animals is visualized as an area of homogeneous radioactivity. The pulp of the molar is of a similar density. However, in the incisor sections, the greatest uptake of radioactivity is seen in the periphery of the pulp.

In the scorbutic animals, the radioactive uptake in the pulp is homogeneous, but of a density greater than that of normal pulp.

Ten-minute survival groups: The general pattern of radioactive uptake as seen in normal animals is one of diffuseness. However, periodontal membrane shows the greatest density. Pulp and supporting bone are seen as areas of similar density, but less than that of periodontal membrane.

No evidence of uptake of radioactivity is seen in any of the radioautographs of the 10-minute scorbutic series. It is interesting to point out that the radiochemical analysis and histologic examination of the other half of the jaw also failed to reveal either substantial radioactivity or ascorbic acid-1-C^{14}.

Five-minute survival groups: The radioautographs of the jaws of both normal and scorbutic animals show a markedly diffuse pattern of radioactive uptake. Periodontal membrane and pulp are seen as the most dense areas. Both these structures evidence radioactive uptake of a similar density.

Saturated animals: In both the 5- and 10-minute saturated animals, the radioautographs show a general distribution of radioactive uptake similar to that of the normal, pair-fed controls. However, in the saturated animals, there is additional evidence of heavy uptake of radioactivity in the supporting bone when compared to the controls.

Summary: Within 5 minutes after intraperitoneal injection of C^{14} labeled ascorbic acid, radioactivity was demonstrated in periodontal membrane, pulp, alveolar periosteum, supporting bone and cortical bone. No activity was evidenced in the enamel or dentin.

Utilizing the radioautograph as a qualitative method of assessing radioactivity, the scorbutic pulps showed a heavier uptake of radioactivity than the corresponding normal pulps. Radioautographic findings would seem to indicate that the periodontal membrane is one of the first tissues to incorporate ascorbic acid. The relatively heavy uptake of radioactivity in the mandibular cortex of the normal animal apparently is a reflection of the growth function. Little uptake of radioactivity is seen in the mandibular cortex of the scorbutic animals.

Histologic Methods.—

Pulp, submaxillary glands and adrenals were removed for histologic examination. In order to avoid subjecting the tissues to decalcification, thereby necessitating a further step which might tend to dissolve some of the

ascorbic acid, the pulp of the incisors was removed in the following manner: Using a bone forceps, the incisors were carefully opened in a longitudinal plane. The pulp, from the region of the basal end of the incisor to the level of the first molar, was exposed and removed with a dental spoon. In so doing, some of the dentin was also removed and is seen in the histologic sections.

Immediately after removal, the various specimens were prepared for the staining of ascorbic acid according to the method of Deane and Morse, as described by Lillie.[3] The specimens were placed for 30 minutes in a modified acetic alcohol silver nitrate solution of Barnett and Bourne.[4] This solution contained silver nitrate 10 grams and distilled water 40 ml. Just before use,

Fig. 7.—Photomicrograph of a section through the pulp and dentin of the mandibular incisor of scorbutic guinea pig sacrificed 5 minutes after injection of radioactive ascorbic acid. This is a section at a level of the first molar. Note the staining of the odontoblasts (*OD*) at the periphery of the pulp. (Silver nitrate stain counterstained with hematoxylin and eosin; × 85.)

10 ml. of glacial acetic acid was dissolved in 50 ml. of absolute alcohol and added to this solution. After 30 minutes, the specimens were placed for 2 hours in an acid-fixing solution containing 50 grams sodium thiosulfate crystals, 1 gram sodium bisulfite, and 100 ml. distilled water. The specimens were then washed overnight in running water. After washing, the material was dehydrated, cleared, infiltrated, and embedded in paraffin according to standard histologic procedure. Serial sections were cut at 4 μ and counterstained with hematoxylin and eosin. In all fixing and staining technics, normal and experimental material was processed in the same solutions at the same time to ensure as accurate control as possible.

Histologic Findings.—

Five minutes after the administration of ascorbic acid to scorbutic animals, there was an intense staining reaction (Fig. 7) in the odontoblastic layer of the mandibular incisor at a level of the first molar. The stain was localized to the cytoplasm of these cells very close to the predentin border

and, in some specimens, the predentin is apparently heavily stained. The odontoblasts presented the characteristic changes seen in avitaminosis C in that, instead of being normally tall columnar cells, they were cuboidal or round in shape. These cells were morphologically indistinguishable from other pulpal cells and were identified as odontoblasts primarily because of their location adjacent to the dentin and their staining reaction. The pulps of the incisors of the non-scorbutic animals (Fig. 8) for the most part showed a staining reaction for ascorbic acid. In a region just posterior to the mandibular first molar, the odontoblasts of the incisors were well-defined, tall, columnar cells having small, round, basophilic staining nuclei and definite elongated cytoplasmic processes.

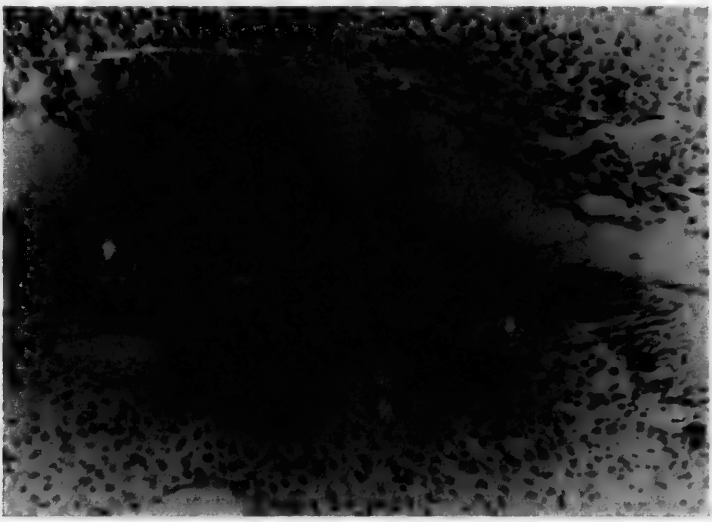

Fig. 8.—Photomicrograph of a section through the pulp of the mandibular incisor of a normal animal sacrificed 5 minutes after the injection of radioactive ascorbic acid. The section is at a level of the mandibular first molar. Note the regular, well-defined odontoblastic layer (*OD*). (Silver nitrate stain counterstained with hematoxylin and eosin; × 85.)

The scorbutic submaxillary glands evidenced an intense staining reaction in their central areas and located in the interlobar and interlobular connective tissue. The acini and ducts showed no stainable material. However, the connective tissue surrounding these structures, including the walls of blood vessels, was intensely stained.

The inferior end of the zona fasciculata and all of the zona reticularis of the adrenals showed a heavy concentration of stainable material. The zona glomerulosa was diminished in width as compared to the non-scorbutic animals but showed no stain. The stain in the zona fasciculata and zona reticularis was again localized to the cytoplasm of the cells and the interlobular connective tissue. The adrenal and submaxillary glands of the control animals evidenced a greater staining reaction than the pulps, as could be predicted from the chemical analysis (Tables I and II, column 3) but much less than similar glands of the scorbutic animals.

In no instance did the dentin of either the scorbutic or non-scorbutic animals evidence any staining reaction for ascorbic acid.

In all groups subsequently sacrificed, the localization of the stain was identical to that just described. However, the intensity of the stain was consistently greater in the groups sacrificed at the later time intervals. The maximum intensity of stain was seen in a 48-hour group previously reported.[5]

TABLE I
INCORPORATION OF ASCORBIC ACID-1-C^{14} INTO ORAL TISSUES OF NORMAL AND SCORBUTIC GUINEA PIGS

ANIMAL	SACRIFICE TIME (MIN.)	ASCORBIC ACID CONTENT (γ/MG. TISSUE)	TOTAL RADIOACTIVITY FOUND (C.P.M./GM. TISSUE*)	RADIOACTIVE ASCORBIC ACID (C.P.M./GM. TISSUE*)
		Pulp		
S 209, 210†	5	N.D.‡	1,500	234
S 212§	10	N.D.	800	60
S 202, 201	15	N.D.	16,300	4,790
S 203, 204	60	N.D.	20,400	9,400
N 214	5	N.D.	1,930	93
N 216	15	N.D.	3,820	377
N 207, 208	60	N.D.	23,800	6,000
		Submaxillary Gland		
S 209	5	N.D.	7,550	67
S 212	10	N.D.	610	90
S 202	15	0.051	8,220	22
S 203	60	0.013	32,600	2,660
N 215, 216	10	0.16	2,340	242
N 207	60	0.93	7,050	1,850
		Tooth		
All scorbutic animals	5-60	N.D.	0	0
All normal animals	5-60	N.D.	0	0

*Since the counting technic was different for the total counts and for the counts in the various fractions these values in column 4 are not directly comparable to those in column 5.
†S is a scorbutic animal and N is a normal animal.
‡N.D.—not detectable by this method, or below a total of 10 γ ascorbic acid in the whole tissue.
§Specimens from tissues of this animal failed to yield adequate radioautographs.

Summary: Five minutes after the intraperitoneal administration of ascorbic acid-1-C^{14}, an intense staining reaction for ascorbic acid was found in the pulps of the incisors, and adrenal and submaxillary glands of scorbutic guinea pigs.

In the scorbutic animals, the ascorbic acid was localized to the cells at the periphery of the pulp. These cells are the original odontoblasts which lost their morphology, but not their potential to produce dentin. Ascorbic acid could not be demonstrated in the formed dentin.

Biochemical Methods.—

Liver, adrenals, submaxillary glands, pulp, dentin, and enamel were dissected from normal and scorbutic animals receiving 11.1 μc of ascorbic acid-1-C^{14} and were analyzed for radioascorbic acid as described later.

TABLE II
INCORPORATION OF ASCORBIC ACID-1-C^{14} INTO NONORAL TISSUES OF NORMAL AND SCORBUTIC GUINEA PIGS

ANIMAL	SACRIFICE-TIME (MIN.)	ASCORBIC ACID CONTENT (γ/MG. TISSUE)	TOTAL RADIOACTIVITY FOUND (C.P.M./GM. TISSUE*)	RADIOACTIVE ASCORBIC ACID (C.P.M./GM. TISSUE*)
		Liver		
S 209†	5	0.68	9,750	2,199
S 212‡	10	0.58	2,376	458
S 202	15	0.32	26,123	5,360
S 203	60	0.68	34,496	6,350
N 214	5	2.24	7,034	1,275
N 215	10	1.55	13.350	4,581
N 216	15	1.97	19.090	5,720
N 207	60	1.33	50,466	4,944
		Adrenal		
S 209	5	N.D.§	17,210	130
S 212	10	N.D.	1,168	22
S 202	15	1.4	95,500	1,240
S 203	60	0.8	72,300	818
N 214	5	1.4	10,800	904
N 215	10	3.1	11,850	224
N 216	15	6.1	21,200	480
N 207	60	2.7	59,500	2,020

*Since the counting technic was different for the total counts and for the counts in the various fractions, these values in column 4 are not directly comparable to those in column 5.

†S is a scorbutic animal and N is a normal animal.

‡Specimen from tissues of this animal failed to yield adequate radioautographs.

§N.D.—not detectable by this method, or below a total of 10 γ ascorbic acid in the whole tissue.

Each tissue was ground with a mortar and pestle, using several milliliters of 0.5 per cent oxalic acid and diethyl ether. Where the tissue weights were small (pulp and adrenal), they were ground with sand and a few crystals of oxalic acid in a small test tube using a glass stirring rod. After grinding, a milliliter of water and ether were added. The mixture was transferred to a centrifuge tube and, after centrifugation, the ether layer was discarded, while the oxalic acid solution of ascorbic acid was saved. The residue was resuspended and re-extracted with several small portions of 0.5 per cent oxalic acid. The combined acid solution was diluted to a known volume and an aliquot titrated with 2,6-dichlorophenolindolphenol to determine the total ascorbic acid content of the tissue. One milliliter of a freshly prepared standard solution containing exactly 5 mg. per milliliter of nonradioactive ascorbic acid was added as a carrier to the remaining solution. The pH was adjusted to 5 by the addition of lead acetate, and any lead oxalate that precipitated was removed by centrifugation. If the solution was turbid, another ether extraction was required to remove the lipids. The solution was immediately placed on a previously prepared and washed Dowex 1 column (28 by 1.2 cm.) in the formate form. The column was washed with water to remove neutral and basic compounds. After collecting some twenty 4.5 ml. fractions, a gradient elution was started. The gradient used was a dropwise addition of 1.5 N formic acid to a 1 L. flask initially containing water. From

each tube a 0.5 ml. aliquot was removed and titrated with standardized phenolindolphenol reagent for ascorbic acid. The tubes were then combined into three fractions. Fraction 1, consisting of the contents of all tubes before the ascorbic acid fraction, contains all the basic, neutral and acids weaker than ascorbic acid. Fraction 2 contains ascorbic acid. Fraction 3, representing contents of all tubes after elution of the ascorbic acid fraction, contains acids stronger than ascorbic acid.

The fractions were then concentrated in a Rinco rotating vacuum concentrator. A few milliliters of water were added, and the fractions were again concentrated to dryness to ensure removal of all the formic acid. Appropriate control analysis using these technics showed better than a 90 per cent recovery of added ascorbic acid and complete removal of the formic acid from the various fractions.

The dried sample was taken up in water and transferred to a combustion flask, and all carbon was then oxidized by the wet oxidation method of Van Slyke-Folch. The carbon dioxide was collected as barium carbonate and isolated on a Tracerlab filtration apparatus. The sample was dried, and immediately counted, using a Nuclear Geiger D-47 tube. All counting data were corrected for self-absorption and for background radiation and corrected to a standard weight of barium carbonate.

Biochemical Findings.—

Within 5 minutes after the intraperitoneal injection of radioactive ascorbic acid, radioactivity appeared in the liver, adrenal, submaxillary gland, and pulp, but not in the hard tissues of the incisor. An appreciable amount of radioactivity (Tables I and II) was found in the ascorbic acid fraction (fraction 2), in the four time intervals studied (5, 10, 15, and 60 minutes). However, we were surprised to find that both the normal and scorbutic guinea pigs had the same approximate total labeling. Thus, it can be seen in Table I, the scorbutic animal, sacrificed at 5 minutes, had a total of 1,500 c.p.m. per gram of pulpal tissue as compared to 1,930 c.p.m. per gram of tissue in the normal. Approximately 20,000 c.p.m. per gram of pulp tissue were found one hour after the injection in both the normal and scorbutic animals.

There are many other examples showing the same approximate total radioactive uptake by normal and scorbutic tissues (see Tables I and II). In order to test this finding of the same radioactive uptake of ascorbic acid in normal and scorbutic guinea pigs, 2 guinea pigs were fed 50 mg. of unlabeled ascorbic acid daily for 21 days prior to the injection of the labeled ascorbic acid. These "pre-saturated" animals showed a considerably smaller uptake of radioactivity in their tissues than was found in comparable tissues of normal or scorbutic animals. The similarity of incorporation of ascorbic acid by both normal and scorbutic animals suggests the possibility that the normal animals, although not showing any clinical symptoms of scurvy, were perhaps somewhat depleted of ascorbic acid. Apparently a 1.5 mg. dose of ascorbic acid was completely used by the tissues of our normal and scorbutic

animals. It is only when we study the uptake of ascorbic acid in the tissues of animals which were presaturated with ascorbic acid that we can observe incomplete utilization of a 1.5 mg. dose.

Table III shows the distribution of the radioactivity in our fractions 1, 2, and 3. It is obvious that only in the liver is the majority of the radioactivity actually ascorbic acid. The compounds in fractions 1 and 3 are unknown as to chemical structure and physiologic significance. Fraction 1, which contains more than half of the total radioactivity, may be predominantly the first oxidation products produced by atmospheric oxygen or by the inadvertent inclusion of traces of metallic ions (copper, especially) during our routine extraction and isolation procedures. If this is true, then the radioactivity in this fraction may be regarded as an artifact. However, since recovery analysis of added carrier is near the theoretical, it is more probable that the radioactivity represents some metabolic product of ascorbic acid oxidation, such as dehydroascorbic acid, 2,3-diketo L-gulonic acid, etc. We are currently contemplating a fuller investigation of the chemical nature of fractions 1 and 3. It is interesting to note that there is a gradual increase in the percentage of the total radioactivity found in fraction 1 with time. Thus, in pulp of the 5-minute sacrifice group, only 30 per cent of the radioactivity is in fraction 1 (the ascorbic acid fraction is 64 per cent), while in the 60-minute sacrifice group as much as 92 per cent of the total radioactivity has been found in fraction 1. It is too early to speculate as to the physiologic significance of fractions 1 and 3.

Radioactivity was lacking in the enamel and dentin.

TABLE III

ASORBIC ACID-1-C^{14} AND NON-ASCORBIC ACID RADIOACTIVE COMPOUNDS EXPRESSED AS THE PERCENTAGE OF THE TOTAL RADIOACTIVITY RECOVERED FROM VARIOUS TISSUES IN REPRESENTATIVE ANIMALS AFTER THE INTRAPERITONEAL INJECTION OF ASCORBIC ACID-1-C^{14}

ANIMAL AND TISSUE	FRACTION 1 (% OF TOTAL COUNTS)	FRACTION 2 (ASCORBIC ACID) (% OF TOTAL COUNTS)	FRACTION 3 (% OF TOTAL COUNTS)
*S 203**			
Liver	4.1	85.7	10.2
Adrenal	84.4	5.9	9.7
Pulp	92.8	7.3	0.9
Submaxillary	59.6	31.9	8.5
Tooth	No radioactivity found		
*N 207**			
Liver	20.1	74.4	5.5
Adrenal	85.4	10.3	4.3
Pulp	74.3	18.8	6.9
Submaxillary	11.1	40.3	48.6

*Sacrificed 60 minutes after the administration of ascorbic acid-1-C^{14}.

Summary: Within 5 minutes after the intraperitoneal injection of ascorbic acid-1-C^{14}, the pulp, submaxillary gland, liver, and adrenal became radioactive. The enamel and dentin were unlabeled. Part of this radioactive material was identified as ascorbic acid-1-C^{14} by isolation from the tissue using column chromatography, and subsequent titration with 2,6-dichlorophenolindolphenol.

Not all of the radioactivity in the various tissues was ascorbic acid. At present, the non-ascorbic acid radioactivity has an unknown chemical structure, and is found in highest proportion in the pulp.

DISCUSSION

In the jaws of both the normal and scorbutic animals, the morphologic patterns, as seen in the radioautographs, correlate closely with comparable histologic sections of the same tissues. Histochemistry discloses localization and distribution of oxidizing substances similar to ascorbic acid within specific tissues. The radioautographs demonstrate the localization and distribution of radioactivity within specific tissues. Chemical analyses identify the oxidizing and radioactive substances, but cannot localize their distribution within the specific tissues.

In our initial study, made with 38 pair-fed controls and 38 experimental animals sacrificed at 2, 8, 24, 48, and 96 hours following intraperitoneal injection of 11.1 μc ascorbic acid, we found heavy radioactive uptake evident in radioautographs made of specimens at all sacrifice periods. Histochemically, ascorbic acid was localized to the cells at the periphery of the pulp. These cells were the original odontoblasts which lost their morphology in the scorbutic animals, but not their potential to produce dentin. The odontoblastic layer appeared as the most active site of metabolism. Ascorbic acid could not be demonstrated in the formed dentin. Part of the radioactivity was identified as ascorbic acid by radioautographs of paper chromatograms.

It therefore became evident that the localization of the vitamin in the various oral tissues was rapid and we attempted to study this phenomenon in the first hour after injection. We have found that, within 5 minutes after injection, the tissues under study were able to incorporate part of a small intraperitoneal dose of the radioactive vitamin.

In earlier studies, Burns and his associates[6] showed, by chemical analysis, that the pattern of distribution of ascorbic acid-1-C^{14} in the scorbutic guinea pigs appears to follow the general distribution of the vitamin already present in the various organs. Our results agree with and extend these findings. The general pattern of distribution of the radioactive vitamin is grossly the same in both normal and scorbutic animals. However, when radioautographic and histochemical studies are compared with chemical findings on the same animal, we see some very definite differences in distribution of ascorbic acid between the normal and scorbutic animal. These differences are all on a sub-organ level.

Our findings support the impression that the two basic processes of growth and repair are intimately associated with ascorbic acid uptake and metabolism.

An expression of the function of growth is suggested by the heavy radioactive uptake as seen in the radioautographs of the expanding mandibular cortical bone in young normal guinea pigs. Absence of radioactive uptake in the cortical bone of the scorbutic animal suggests the possibility of a cessation of growth in this area. Both the smooth outline and homogeneous density

in the normal periodontal membranes, as seen in the radioautographs, suggest that this tissue is not functioning at an abnormal stress level. In contrast, the irregular outline of the scorbutic periodontal membrane, coupled with the heavy radioactive uptake in the peripheral areas of this tissue, seems to indicate heavier demands for ascorbic acid. This impression is amplified in comparing the pattern of radioactive uptake in the epiphytic areas in supporting bone of the scorbutic group. The extensive ascorbic acid metabolism in this area is indicated by the fact that 92 per cent of the radioactivity in the pulp is no longer ascorbic acid after 1 hour. Histologic studies of this area in the later time periods always showed the repair and growth processes in progress.

As a result of comparing uptake of radioactivity at the various sacrifice levels through the entire study, in both normal and scorbutic animals, the suggestion is made that the periodontal membrane is one of the first tissues to incorporate ascorbic acid. When the periodontal membrane was removed, its macerated condition did not lend itself to histologic examination. A sufficient volume of periodontal membrane could not be made available for a corroborative chemical analysis. We hope to obtain sufficient periodontal membrane to complete our studies.

The pulp, by virtue of its relatively heavy concentration of radioactivity, also appears to be a tissue of active ascorbic acid metabolism. Although the distribution of density in the radioautographs is generally homogeneous within the pulp, the relatively greater density along its periphery suggests that the odontoblastic layer has still greater ascorbic acid metabolism than other portions of the pulp. Similarly, histochemical findings indicated that the cells adjacent to the dentin in acutely scorbutic animals stained intensely with the silver nitrate method for ascorbic acid, while cells of the control animals, for the most part, showed no demonstrable staining reaction even though the staining procedure of both tissues was identical. It should again be pointed out that, in the radioautographs of the pulp, not all density areas seen are due to ascorbic acid radioactivity per se, but the larger amount of radioactivity appears to be of unknown chemical composition. We believe this emphasizes the importance of this vitamin in maintaining the integrity of the pulp, and production of dentin by the dentin forming cells at the periphery of the pulp.

Wolbach and Bessey[7] regard the question as to whether or not the odontoblasts disappear and degenerate in ascorbic acid deficiency or simply lose their morphology as unsettled. However, Thorell and Wilton[8] concluded that the change induced by avitaminosis C is one in which the differentiated odontoblasts revert to a more immature cell type. In our study, the cells at the periphery of the pulp show an intense staining reaction for ascorbic acid in the relatively short period of 5 minutes after the administration of ascorbic acid. It is our impression that these cells are the original odontoblasts which, because of a lack of ascorbic acid, have lost their morphology and have become indistinguishable from other pulpal cells. The potential to produce

dentin, however, was still inherent and became evidenced with the administration of ascorbic acid. It is not suggested that these are the only pulpal cells which metabolize ascorbic acid.

Although the histologic findings in the scorbutic animals are in agreement with those evidenced by radioautography and biochemistry, the smaller amounts of stainable material in the normal were wholly unexpected. At present, no explanation is offered for the fact that the normal tissues with their higher concentration of ascorbic acid (as determined by chemical analyses) failed to exhibit characteristic amounts of silver staining. In spite of this, we believe the histologic findings observed in the scorbutic animals are accurate in that they are corroborated by the radioautographs and chemical analysis.

Chemical analysis also revealed an early uptake of radioactivity in the pulp. There was a gradual increase in total radioactivity and labeled ascorbic acid in pulpal tissues from 5 minutes to 60 minutes after the administration of the vitamin. Even more interesting is the finding that the adrenal, submaxillary gland, and especially the pulp have a large amount of non-ascorbic acid radioactivity. This non-ascorbic acid radioactivity probably represents some physiologic change (and not an artifact), since recovery of added carrier ascorbic acid precludes the possibility that it represents an oxidation product of the vitamin by atmospheric oxygen during the analysis. The possibility has not been eliminated that the non-ascorbic acid radioactivity may be dehydroascorbic acid (another form of the radioactive vitamin) which can be readily utilized by the tissues.

It is interesting to point out that the tissues which show specific pathology in scurvy (pulp, periodontal membrane, bone, etc.) have a high proportion of this non-ascorbic acid radioactivity, while the liver, although of course requiring the vitamin, does not show as profound a pathology during scurvy and has a low proportion of these compounds. This relationship may be a reflection of physiologic utilization of ascorbic acid to repair the damage done during the scorbutic period.

Parenthetically, in all 96 samples analyzed (5 minutes to 96 hours) no more than a few counts of radioactivity were found in the hard structures of the tooth. We assume this minor amount of radioactivity to be contamination due to incomplete removal of all pulpal tissue. This finding is in agreement with all of the radioautographs, which consistently failed to show any radioactive uptake in enamel and dentin, and with the lack of histochemical evidence demonstrating the vitamin in the enamel or dentin. Although our results generally agree with those of Burns and co-workers[6] the finding of no radioactivity in the enamel and dentin of our animals appears to disagree with the Burns report that the tooth has a very high uptake of radioactivity. However, if we can assume that Burns included the pulp and perhaps some peridontal membrane in his tooth preparation, then the difference in results is resolved.

Salomon,[9] in a study of the kinetics of ascorbic acid metabolism in normal and scorbutic guinea pigs, showed that the catabolic rate is unchanged in scurvy. The scorbutic animal degrades the same definite proportion of ascorbic acid each day as does the normal. By chemical analyses, we have shown approximately the same total uptake of total radioactivity and radioactive ascorbic acid in tissues of both the normal and scorbutic guinea pigs. This indicates, as Salomon has pointed out, that the same rate of ascorbic acid metabolism exists in the scorbutic animal as appears in the normal. This finding is in disagreement with the general belief that a deficient animal will conserve or more efficiently use the missing essential nutrient than the normal animal.

SUMMARY AND CONCLUSION

On the basis of radioautographs, histochemical staining technics and chemical analysis we made the following observations.

1. Within 5 minutes after the intraperitoneal injection of 1.5 mg. of ascorbic acid-1-C^{14} containing 11.1 μc of activity, radioactivity was demonstrated in periodontal membrane, pulp, alveolar periosteum, supporting and cortical bone, liver, adrenal and submaxillary gland of both normal and scorbutic guinea pigs.

2. Using these same technics, we consistently failed to find any radioactivity and/or ascorbic acid in the enamel and dentin in either normal or scorbutic guinea pigs.

3. Part of the radioactive material was identified as ascorbic acid-1-C^{14} by isolation from the tissue and subsequent titration with 2,6-dichlorophenolindolphenol.

4. Histochemistry localized the ascorbic acid in the pulp to the cells at the periphery which were probably the original odontoblasts. These cells had, in the case of scorbutic animals, lost their morphology but not their potential to produce dentin. Radioautographs of the pulp showed a homogeneous uptake of radioactivity in the normal. However, in the scorbutic pulp, a zone of greater uptake was seen in the area closest to the dentin.

5. From examination of the radioautographs, it appears that the periodontal membrane was one of the first oral structures to incorporate the radioactive vitamin. Our results also indicate that this tissue had a high concentration of radioactivity.

6. With the exception of enamel and dentin, there was localization of radioactivity in all of the tissues studied in both normal and scorbutic animals. However, there was variation in the pattern of distribution of radioactivity when normal and scorbutic tissues were compared on a sub-organ level.

7. Not all the radioactivity in the various tissues was ascorbic acid. At present, the non-ascorbic acid has an unknown chemical structure and is found in highest proportions in the pulp.

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Erratum

In the article, "Variability of Tooth Formation," by Stanley M. Garn, Arthur B. Lewis, and Demarest L. Polacheck, in the January-February, 1959, issue of the JOURNAL, the center section of Fig. 5 on page 143 should have been labeled "Root Formation" instead of "Root Completion."

RESEARCH ANNOTATIONS*

RELATIONSHIP OF CARIES INCIDENCE IN DECIDUOUS AND PERMANENT DENTITIONS

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IT WOULD be of value in dental diagnosis to know whether the incidence of carious lesions in deciduous teeth permits one to predict the future caries activity in the permanent dentition. In Baja, school children are examined yearly for caries. In the following study, the records of 97 of these school children, examined in 1950-1951 at the age of 5 and again in 1955-1956 at the age of 11, were utilized. A comparison of data from these records is seen in Table I.

TABLE I

COMPARISON OF CARIES FORMATION IN THE DECIDUOUS AND PERMANENT DENTITION AT 5 AND AT 11 YEARS OF AGE

1. Number of deciduous teeth with caries	0	1	2	3	4	5	6	7	8	9	10	11	12	16
2. Number of school children with carious deciduous teeth, 1950-1951	23	12	14	10	7	7	6	5	6	1	–	4	1	1
3. Number of school children with noncarious permanent dentition, 1955-1956	12	1	1	0	0	0	1	0	0	0	–	0	1	0

This shows that of the 97 children, 23 had no caries at the age of 5 years, 12 had one carious deciduous tooth, 14 had 2, 10 had 3, and so on. Of the 23 children who had no caries at this age, 12 (52 per cent) had not developed caries by the second examination. Of the other 74 children with caries at the age of 5 years, only 4 (5.4 per cent) were free of caries at 11 years of age. There was only one child with a noncarious permanent dentition among both the 1 and 2 dmf index groups. Infrequently, children showed a caries-free permanent dentition following a markedly carious deciduous dentition (dmf = 12). Of the 16 children with caries-free permanent dentition, 12 (75 per cent) had caries-free deciduous teeth.

If we compare the caries incidence of these children at age of 5 years with the incidence at age of 11 years, we see the probability that 75 per cent of the children with caries-free permanent teeth will have had caries-free deciduous teeth. The chance that this relationship will not occur is much less than 0.1 per cent. From this we can deduce the fact that children with caries-free deciduous teeth will probably have caries-free permanent teeth. This statement should hold true as long as environmental factors have not changed significantly.

Frenzel (Deutsch. Monatschr. f. Zahnheilkunde 51: 437, 1933) has found that there is a correlation between the state of health of first permanent molars and that of deciduous teeth. He pointed out that this correlation was not due to lack of treatment of deciduous carious teeth resulting directly in caries of the permanent dentition.

Summary.—Examination of 97 children aged 11 years, whose caries records taken at age of 5 years were accessible, showed that 12 of the 16 children with noncarious permanent teeth at the age of 11 years had noncarious deciduous dentition at the age of 5 years. There appears to be some measure of correlation between caries formation in the deciduous dentition and that in the permanent dentition.

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*Short notes with or without illustrations submitted to this section are reviewed and published promptly on one page of the JOURNAL in accordance with the previously announced editorial policy (*J. D. Res.* 37: 373, 1958).

COLLECTION AND STERILIZATION OF SUBMAXILLARY-SUBLINGUAL SALIVA

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A METHOD was desired for obtaining sterile submaxillary-sublingual saliva in order to carry out bacteriologic experiments. Regardless of variation in collection method, viable microbes, which contaminated the secretion outside Wharton's duct, could not be eliminated. Attention was therefore centered on inactivating these microbes without significantly altering the enzyme systems. Ultraviolet irradiation of thin saliva films was selected after several experiments.

Methods and Results.—A modified Pickerill segregator as described by L. H. Schneyer (J. D. Res. 33: 683, 1954 [Abst.]) was cast in aluminum from wax patterns prepared on plaster models. The models were made on alginate impressions of the sublingual area. The appliance contained a short collecting tube integral with the casting. Tygon tubing was attached to the casting, then the entire apparatus was wrapped in aluminum foil and autoclaved at 121° C. for 30 minutes. Isolated submaxillary-sublingual saliva was collected in sterile test tubes. The saliva was transferred immediately from the sterile test tubes into sterile stainless steel trays, 4 in. by 8 in., creating a film to a 1.2 mm. maximum thickness. These trays were then inserted into a box containing an ultraviolet tube (Westinghouse G 15 T 8 germicidal lamp with a peak output of 2537 A) and irradiated at a distance of 5 inches from the source for 5 minutes. The saliva was tested for sterility by incubating 1 ml. aliquots on blood-agar medium at 37° C. for 24 hours. Two blood agar plates were employed, one being incubated aerobically, the other anaerobically.

Evaluation of the effect of irradiation on the amylolytic activity of the saliva was determined on sterilized experimental samples (Koch, F. C.: Practical Methods in Bio-Chemistry, ed. 2, 1937, Baltimore, William Wood & Co., pp. 77-79). The saliva was assayed immediately following irradiation with the inclusion of proper controls. Results seemed to indicate that amylolytic activity was reduced, but the amount of reduction was less than the experimental error of the test procedure. Tests run at daily intervals to evaluate the effect of storage (5° C.) on amylolytic activity indicated no change over 7 days.

Conclusions.—Ultraviolet irradiation of collected submaxillary-sublingual saliva, as performed in this work, supplies quantities of saliva free of viable microbes and with minimally altered enzyme activity. The proportion of submaxillary-sublingual secretion in mixed saliva is high (Schneyer, L. H., and Levin, L. K.: J. D. Res. 34: 725, 1955 [Abst.]), thus greater study of its characteristics and properties will contribute to a better understanding of oral ecology.

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EFFECT OF RATE OF FREEZING ON THE PRESERVATION OF VIABLE TOOTH GERMS

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THE effects of rates of freezing on the preservation of tooth germs, pretreated to withstand the harmful effects of low temperatures, were investigated by varying the immersing time of the tissues into a single freezing agent, liquid nitrogen. Previous work by the authors had shown the superiority of solid carbon dioxide (dry ice)—ethyl alcohol mixtures (–78° C.) over liquid nitrogen (–198° C.) as a preserving agent in maintaining viability of the tooth germs when each agent was used to freeze the tissues as rapidly as possible. The results were assumed to be due to either the slower rate of freezing at –78° C. or to the higher freezing temperature of the dry ice alcohol mixture.

Incisor and molar tooth germs from rats, approximately 18 days fetal age, were removed aseptically and placed into glycerol-physiological saline (Tyrode) solutions (glycerol concentrations 20 per cent and 30 per cent by volume) for 15 to 30 minutes. The tissues were then transferred to sterile wood splints and sealed into thick-walled test tubes in such a way that the tooth germs did not adhere to the glass. The tubes were slowly lowered into the freezing agent, liquid nitrogen, at the rate of 10 minutes per inch, until they were completely immersed in the freezing fluid. Ten minutes after complete immersion, the tissues were removed for thawing. The tissue-bearing splints were quickly withdrawn and immediately plunged into physiological saline at about 38° C. After thawing the glycerol was removed from the tooth germs by washing them for 10 minutes in each of 3 changes of Tyrode solution.

All tissues were cultured in Carrel flasks for observation of viability and ability to resume growth. Since the object of culturing was to determine the state of vitality of the tooth germs at the time of explanting, chiefly by the extent of cellular outgrowth from the tissues, the cultures were maintained for only 7 days. The medium used was fresh frozen chick plasma, fresh frozen chick embryo extract, and horse serum in ratios of 1:1:2. The tissues were incubated at 36° C. and examined daily.

The tooth germs frozen slowly by delayed immersion into thhe liquid nitrogen appeared as viable as, and grew to somewhat larger size than, the controls frozen by rapid immersion into solid carbon dioxide-alcohol mixtures. The extent of cellular outgrowth from the liquid nitrogen slowly frozen tissues was greater than from the dry ice cooled controls, probably indicating that more cells survived the slower freezing method, although cytologically the cells appeared equally healthy after both methods. These results are interpreted as indicating that the rate of freezing, and not the freezing temperature, is of greater significance in the freezing and preservation of oral tissues for storage purposes. Experiments are under way extending these methods to an examination of tooth germs frozen by delayed immersion into dry ice mixtures at –78° C.

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A CAPILLARY NETWORK IN THE ODONTOBLASTIC LAYER OF DEVELOPING TEETH

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LONG Evan's rats and Syrian hamsters, ranging in age from 10 days to 11 months, were perfused with India ink. The fluid was introduced into the circulation of the living, anesthetized animal by perfusion of the left ventricle. In the developing molar tooth of animals, 10 to 20 days old, the ink was deposited in what appeared to be a capillary network within the odontoblastic layer, and closer to the dentin than the nuclei of the odontoblasts. The deposits were regular and interrupted around the periphery of the pulp chamber and were perpendicular to the odontoblasts, displaying connecting links with the deeper pulp vessels. These deposits of ink appeared to be identical in form with another network which was located along the outer margins of the ameloblasts. The latter deposits could not be observed when the ameloblasts lost their characteristic appearance at the completion of enamel formation. Identification of these areas as capillaries was confirmed by staining uninjected specimens with hematoxylin and eosin, and Mallory's trichrome. In the stained sections, erythrocytes are visible in the areas in which the India ink was deposited.

In the molars, the odontoblastic capillaries could only be observed in the coronal portion of the tooth, within the area bounded by forming enamel. Apical to this area, the capillaries related to the outer margins of the ameloblasts are present but not the odontoblastic capillaries. The outer capillaries completely surround the tooth bud. This may be interpreted as an indication that the odontoblastic capillaries are associated with the formation of dentin. It is possible that when the enamel is laid down, the odontoblasts are cut off from the ameloblastic capillaries in the enamel area. In the root area, since there is no enamel the outer capillaries can supply the odontoblasts and there is no need for vascularization from the pulp.

In the incisors, since dentin is continuously laid down, it would be expected that the odontoblastic capillaries would persist. This was found to be the case in the oldest rats and hamsters examined, 11 months of age. They are present on both the labial and the lingual surfaces and extend down to the apical area of the incisors. This would be necessary as the enamel extends to the apical region of the labial, blocking the outer ameloblastic capillaries. They are necessary on the lingual as there are no ameloblastic capillaries.

Shortly after eruption of the molars, the pulpal vessels extend only up to but not into the odontoblastic layer. This approximately coincides with the termination of dentin formation.

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*Postdoctoral Fellow.

EFFECTS OF EXTERNAL ENVIRONMENT ON MUCOSA OF THE HAMSTER'S POUCH

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EPITHELIA can be altered with many different stimuli (chemical, mechanical). It occurred to us to investigate the effects of exposure on the mucosa of the hamster cheek pouch.

An incision was made in the neck through the skin and into the cavity of the cheek pouch. A large area of mucous membrane of the pouch was then pulled through the incision and sutures were made between the edges of the cut skin and the wall of the exteriorized pouch. A sac, approximately 0.5 inch in diameter, with the mucous epithelium exposed to the outside environment and with an adequate intact blood supply was thus created. This sac was very pink in appearance due to the vessels under the thin mucous epithelium. In time the pouch became smaller and the reddish hue disappeared to some extent. The paler appearance was due to changes in the epithelium which looked drier and more keratinized and not to a reduced blood supply, as shown by injection studies.

Biopsies were taken from the normal and exteriorized pouches and histologically prepared. The epithelium of the normal pouch consists of several layers of superficial cornified cells; a layer of cells with a few granules, and a four or five layered stratum malpighii. The cells of the latter stratum contain large nuclei which stain intensely and nearly fill most of the cells. The small amount of cytoplasm is clear and vacuolated.

By 15 days after exteriorizing the pouch, great changes have taken place in the exposed epithelium. The malpighii stratum has thickened four or five times. The cells adjacent to the dermis are small and cylindrical in shape and have deeply staining nuclei. As the cells are pushed peripherally, they increase several times in size and assume a polyhedral shape. They form a definite prickle cell layer with typical intercellular bridges. The granulosum is represented by several layers of flattened cells which contain granules. The transition from the granulosum to the stratum corneum is very abrupt. Although the corneum is approximately twice as thick, its structure and density are similar to that in the normal mucosa. The corneum of both the normal and exposed epithelium contains abundant disulfides as demonstrated by the Barrnett-Seligman technic.

Under the circumstances of this exposure, greater opportunity exists for water loss and the epithelium is subjected to wear and contacts which necessitate more protection for the underlying tissues. Accordingly, all the layers of the epithelium increase in thickness and some change in character or a metaplasia occurs to meet the effects of environment.

A histochemical analysis of the sequence of events is being made to interpret the mechanism of this environmental factor on the tissue.

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AGING, DIET, AND PERIODONTAL LESIONS IN THE HAMSTER

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IT has been demonstrated by Mitchell (J. A. D. A.: 49: 177, 1954) that a soft, fine-particle, carbohydrate diet in the hamster promotes a periodontal destruction exceeding that which occurs on a diet of coarse laboratory chow. Since this does not occur when finely ground regular chow is fed, it appears that the fineness, per se, is not responsible for the increased destruction. The effect appears not to be due to nutritional deficiency, as measured by increase in body weight, but apparently to a local reaction to mucinous plaques which are formed at the gingival margins of the teeth of animals on a soft carbohydrate diet.

A study was made to determine the effect of aging on the susceptibility of the periodontium of hamsters to this kind of dietary insult. Hamsters were placed on an ad libitum diet of half powdered milk and half sugar at 20 days, 100 days, and 250 days of age. The control animals were fed regular laboratory pellets. After 30 to 60 days, the animals were sacrificed and sections of the jaws examined in cross and sagittal section after staining with hematoxylin and eosin. Periodontal lesions were identified by increased downgrowth of the epithelial attachment, pockets, breakdown of the interproximal papillae, and inflammation. At the beginning of the experimental period, all the animals had the molars in one quadrant of the upper jaw reduced 0.5 to 1.0 mm. by grinding since this might enhance the destructive effect of the carbohydrate diet.

Examination of the sections revealed no effect that could be associated with the occlusal reduction on either the diet or control animals. Furthermore, there were no alterations in the thickness of the epithelium or degree of keratinization. Since the animals on diets for 60 days and 30 days differed only in degree, this report is confined to the 30-day animals.

Both the control and the diet animals started at 20 days of age were found to be practically free of periodontal lesions. The only visible result of the carbohydrate diet was an accumulation of a basophilic debris in the gingival crevices. The maximum effect of this debris was a partial flattening of the interproximal papillae and a broadening of the gingival crevice. There was no increase in downgrowth of the epithelial attachment, no pocket formation, and no increase in inflammation.

The 100-day-old animals on coarse diets suffered some downgrowth of the epithelial attachment, inflammation, and some destruction of the interproximal papillae. In the 100-day animals on soft diets, the downgrowth of the epithelial attachment and destruction of the interproximal papillae were increased in extent as compared with the 100-day controls. The most severe damage was visible interproximally, and least on the mesial of the first molar and distal on the last molar.

The 250-day coarse diet animals displayed marked downgrowth of epithelial attachment, breakdown of interproximal papillae, inflammation, and pocket formation in a similar pattern but to a greater extent than the 100-day coarse diet animals. Examination of the carbohydrate diet animals of 250 days revealed much more severe destruction. Pockets up to one half the length of the roots were visible interproximally, buccally, and lingually. The relative amount of destruction of the 250-day soft diet animals, as compared with the coarse diet animals of the same age, was in excess of that of the other two younger age groups. Despite the great damage, the collagen bundles in the intact portion of the periodontal membrane appeared normal. This, and the normal weights of the animals, indicated a local rather than systemic or nutritional effect.

Thus, in the hamster, there is an increase of periodontal destruction with increasing age on the standard coarse laboratory diet. This apparently predisposes the older animal to a greater susceptibility to increased periodontal destruction from a dietary insult in the nature of a refined carbohydrate diet, as compared with the more intact periodontium of a younger animal.

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*Postdoctoral Fellow.

EFFECTS OF DIFFERENT PRESERVATIVES AND SILICONE DENTIFRICES ON CONTACT ANGLE OF WATER ON TOOTH ENAMEL

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THE effects of preservatives and silicone dentifrices on the surface reactivity of tooth enamel can be determined by "contact angle" measurement, for example, the angle of contact a drop of liquid makes with a solid surface. The tooth was cleaned with a rubber cup and fine pumice, was air dried, wiped clean with lens paper, and placed on an adjustable platform within a cuvette. The cuvette was covered and water was placed in the bottom to maintain a saturated atmosphere. Stereomicroscope (13×) was used in a horizontal position. Horizontal and vertical dimensions of the drop were readily measured with a fine scale ocular micrometer, the vertical first, as it had been observed to change with time.

Mack (J. Phys. Chem. 40: 159-167, 1936) has established the spheroidal segment technic for determining the contact angle as: $\theta = 2 \text{ TAN}^{-1} (H/X)$, where θ is the contact angle, H is the height of the drop, and X is the radius of the base of the drop.

Contact angles were measured on 101 teeth in three groups: (A) 110 observations on 10 freshly extracted teeth, (B) 180 observations on 10 teeth preserved in 10 per cent formalin, and (C) 405 observations on 81 teeth preserved in Zephiran Chloride (Benzalkonium chloride, 12.8 per cent diluted to give 1:1,000 strength). Seventy-five of the 101 teeth came from females and 75 were anteriors. Over 60 per cent of the patients were under 30 years of age.

Contact angle readings of Group A (mean 70.10 degrees, range 66.08 to 77.68 degrees) were significantly lower than Group B (mean 80.35 degrees, range 75.21 to 85.38 degrees, t 3.96, probability less than 0.05), and Group C (mean 78.90 degrees, range 51.00 to 98.50 degrees, t 6.43, probability less than 0.05). These preservatives definitely altered the nature of the enamel surface.

Three groups of 10 teeth each were studied before and after brushing with different dentifrices. Mean contact angle differences (experimental minus control) were significantly different from zero at 5 per cent level for A, water (9.8 ± 5.0), (B) control tooth powder (N. F.) (9.6 ± 5.0), and (C) silicone tooth powder* (17.0 ± 5.9). Two silicone tooth pastes A† and B‡ were similarly tested on two groups of 10 teeth. A difference (probability less than 0.05) was observed in the teeth before and after brushing with silicone pastes. The concentration of silicone present in the preparation appears to increase the differences between the control and experimental readings.

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*Silicone tooth powder: 8 grams tooth powder (N. F.) to 1 c.c. silicone DC200, 350 cs.

†Silicone paste A: 10 grams tooth powder (N. F.); 5 c.c. silicone and 5 c.c. glycerol.

‡Silicone paste B: 10 grams tooth powder (N. F.) to 8 c.c. silicone.

IADR SECTIONAL MEETINGS

IOWA AND MINNESOTA SECTIONS

The Spring meeting of the Iowa Section of the International Association for Dental Research was held in conjunction with the Minnesota Section at Rochester, Minn., on Dec. 11, 1958, 7:30 P.M.

The Research Reports were presented in the following order:

1. Department of Operative Dentistry, State University of Iowa, sponsored by Dr. James H. Wick:

   a. Evaluation of Cavity Liners Under Silicate Cement.—Dr. Wallace W. Johnson.
   b. Manipulative Variables of Thiokol, Silicone and Alginate.—Dr. Ernest A Sahs.

2. Department of Stomatology:

   a. Effects of Procaine on Respiration in the Dental Pulp.—Christian Schwabe, D3.
   b. Cholesterol Content of Bovine Dental Pulp.—James J. Stickley, D3.

3. Department of Crown and Bridge, State University of Iowa, sponsored by Dr. Ray V. Smith:

   a. The Baking of Low Fusing Porcelain to Gold.—H. F. Sawyer, Lt. Col., DC, USA.

4. Department of Orthodontia, State University of Iowa, sponsored by Dr. Ernest H. Hixon:

   a. Age Changes in Facial Profiles.—Dr. William Bowker.

5. Child Welfare Research Station:

   a. Age Changes in Arch Dimension.—Dr. Virginia Knott.

At the conclusion of the Program, the Group adjourned to the Students' Lounge where coffee and doughnuts were served.

INDIANAPOLIS SECTION

The Indianapolis Section of the International Association for Dental Research held a meeting January 23 in the Student Union Building at 4:00 P.M. The program had a wide range of interest. Short papers were presented by members of the local organization as follows:

The Possibility that Fluoride in Bone Can Increase the Uptake and Retention of Long Lived Stronium.—Dr. W. E. Rogers.

Fused Porcelain Veneers.—Dr. J. F. Johnston.

Tumor Induction Studies as Related to Dental Materials.—Dr. D. F. Mitchell, Shirley Shazer, and Dr. G. B. Shankwalker.

Mandibular Growth Patterns in the Preadolescent.—Dr. C. J. Burstone.

The Effect of Topical Application of Stannous Fluoride in a Fluoride Area.—Dr. J. C. Muhler.

These talks were followed by a dinner at 6:00 P.M. and an address by D. Kenneth Kohlstaedt, Director of Clinical Research at the Eli Lilly Company. He spoke on "Current Trends in Clinical Research."

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